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Molecular Biology of Floral Evocation in *Lolium temulentum*

by

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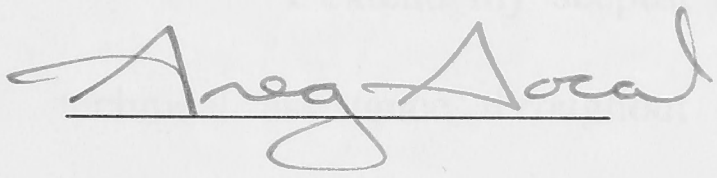
CANDIDATE'S STATEMENT

The research reported in this dissertation is original and has not been submitted for any other degree at this or any other university. The work was my own with the following provisos:

Technical assistance was provided by Cheryl Blundell for many of the *in situ* hybridisation and immunolocalisation experiments as well as during library screening for the *Lolium* CDC2a cDNA.

Technical assistance was provided for library screening and sequencing of the *Lolium* GAMYB cDNA by Cheryl Blundell and Robyn Watts respectively. Expression of *Lolium* GAMYB in *Lolium* de-embryonated half grains was performed by Robyn Watts and Frank Gubler.



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GFW Gocal

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ABSTRACT

Currently an exciting and important area of developmental biology involves the study of the changes in gene expression occurring at the shoot apex during floral evocation. *Lolium temulentum* presents an ideal system to study these changes since it is specifically inducible by a single long day (LD), remaining vegetative in short days (SD's). As well as providing a good vegetative control, this specific induction creates an effective time zero. In *Lolium* the vegetative and floral developmental programs are both spatially (leaf primordia versus spikelet sites, respectively) and temporally separated (SD versus LD, respectively).

In many florally inducible systems, including *Lolium*, the earliest visible change occurring within the shoot apex is an increase in cell division. CDC2, a kinase required for cell division, was used to examine the increased potential for cell division during the vegetative to floral transition. PCR-based northern blotting, immunolocalisation (using an anti-wheat CDC2 antibody) and *in situ* mRNA analysis (using riboprobes specific for the *Lolium CDC2a* homologue) confirmed this gene is highly up-regulated within 24 h after the end of the LD. This correlates well with the time at which the rate of primordium production begins to increase (Evans and Blundell, 1996).

Also within 24 h of the end of the LD, the expression of two APETALA1-like genes, termed *Lolium temulentum MADS1* and *LtMADS2*, increases in the shoot apical meristem of *Lolium* as determined by PCR-based northern analysis and *in situ* mRNA analysis with gene-specific riboprobes. Both genes were expressed in the vegetative, spikelet and floret (floral) meristems, however, later in development LtMADS1 was expressed in all organs of the floret with LtMADS2

being excluded from the sterile organs of the perianth. *LtMADS2* is most similar in sequence and expression pattern to *ZAP1* from maize.

Another gene involved in floral development is *LEAFY*, therefore, a conserved fragment of this gene was isolated from *Lolium*. Up-regulated expression of LtLFY, visualised by either *in situ* hybridisation or by immunolocalisation (using an anti-*Arabidopsis* LEAFY antibody), was not detected until well after the induction event (LD XII). This gene was expressed within glume primordia and unlike its cognate homologues from other species was expressed within the spikelet (inflorescence) meristems. The up-regulated expression of LtLFY is late compared to when these genes are activated in *Sinapis* or *Arabidopsis*.

The *Lolium GAMYB* homologue, a transcriptional regulator from the GA-response pathway, was isolated from a florally induced (LD III) shoot apex PCR-based cDNA library. Like its homologues from barley and rice, its expression in seeds is up-regulated by GA. The *Lolium GAMYB* gene is also expressed in the shoot apex throughout development (from SD vegetative to LD XXX floral apices), however, its expression increases soon after LD induction. Highest expression is detected within the spikelet sites of double ridge stage apices. Thus within the shoot apex, GAMYB may respond to increases in GA level by inducing the expression of flowering genes.

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ABBREVIATIONS

ATP ..... adenosine triphosphate

bp ..... base pair

BSA ..... bovine serum albumin

CDC2 ..... Cell Division Cycle

cDNA ..... cloned deoxyribonucleic acid

DEPC ..... diethyl pyrocarbonate

dicot ..... dicotyledonous species

DIG ..... digoxygenin

dNTP ..... deoxynucleotide triphosphate

DTT ..... dithiothreitol

EtOH ..... ethanol

F.W. ..... fresh weight

h ..... hour

GA ..... gibberellin

IAA ..... isoamyl alcohol

LD ..... long day

min ..... minute

monocot ..... monocotyledonous species

NAD ..... nicotinamide adenine dinucleotide

mRNA ..... messenger ribonucleic acid

OAc ..... acetate

ORF ..... open reading frame

PCR ..... polymerase chain reaction

pfu ..... plaque forming unit

RACE ..... rapid amplification of cDNA ends

rpm ..... revolutions per minute

SD ..... short day

SE ..... standard error

UTR ..... untranslated region

CHAPTER 1

INTRODUCTION

The majority of plant species reproduce sexually, therefore the timing of their vegetative to floral transition is of paramount importance, since it is a major factor determining the ability of a plant to successfully set seed as well as its ability to outcross. Directly or indirectly, the seeds and fruits produced through this process are the basis of the world's food supply. Therefore, study of this transition may have substantial consequences. In the future, basic research in this area will provide the basis for manipulation and control of the reproductive process in higher plants. For some pasture grass species, flowering can be detrimental since it causes the production of a lignified stem which is largely indigestible. Inhibition of flowering in these, and in weed species, might also be accomplished through better understanding of the flowering process.

Flowering may be divided into the component processes of competence, induction and development (see Figure 1.1). The ability of a plant to respond to an inductive environmental stimulus is termed competence. This is acquired by development through a juvenile growth phase to become a mature plant - one which is capable of flowering. Such juvenility is presumably to prevent flowering until a plant is large enough to adequately supply developing reproductive tissues (floral buds, seeds and fruits) with assimilate (see refs. cited in Zimmerman *et al.*, 1985 and Pharis *et al.*, 1989).

Induction refers to a plant's response to an environmental stimulus leading to floral development, with photoperiod and temperature often playing a dominant role in this process. Generally photoperiodic stimuli are perceived by the

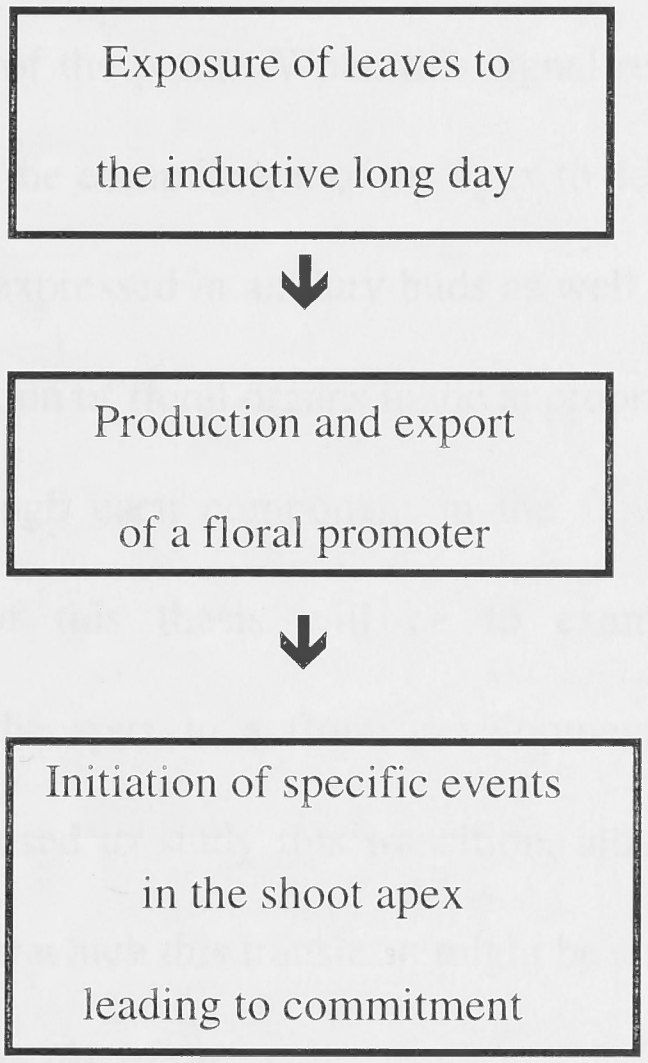


Figure 1.1 Components of the flowering process in *Lolium*. (Adapted from Bernier, 1986)

leaves, whereas vernalisation or thermoinduction is perceived by tissue which has potential for cell division (reviewed in King and Gocal, 1997; Metzger, 1988). During photoperiodic induction, a florigenic signal is passed from the leaves to the growing point(s) of the plant. When this signal reaches the apical meristem (shoot apex), evocation, the commitment of the apex to develop florally follows. The floral program may be expressed in axillary buds as well as in the terminal bud, eventually causing the initiation of floral organs in the appropriate position, number and size.

Although each component in the flowering process is important, the primary focus of this thesis will be to examine evocation, the irreversible commitment of the apex to a floral developmental program. Herein, molecular techniques were used to study this transition, although this is clearly not the only point of view from which this transition might be studied.

1.1 *Lolium the Plant*

Flowering of the grass *Lolium temulentum* L. strain Ceres has been studied over the last 40 years at the Canberra phytotron (reviewed by Evans, 1969 and Evans and King, 1985). Floral induction/evocation in response to a single long day (LD) photoperiod has enabled *Lolium* to become a model system. Although sensitivity to the LD increases with plant age, 6 week old seedlings are induced to flower with a single LD (Evans, 1960). Strict daylength control also allows the vegetative condition to be determined with more certainty. Single LD induction is highly effective when given as a very low fluence extension of an 8 h SD (see Figure 1.2), minimising the photosynthetic effects of the LD. The critical LD for a single inductive cycle is between 12 and 16 h (Evans, 1969), depending on plant age and light conditions. Vernalisation has no effect on flowering of this annual LD plant (Evans, 1969).

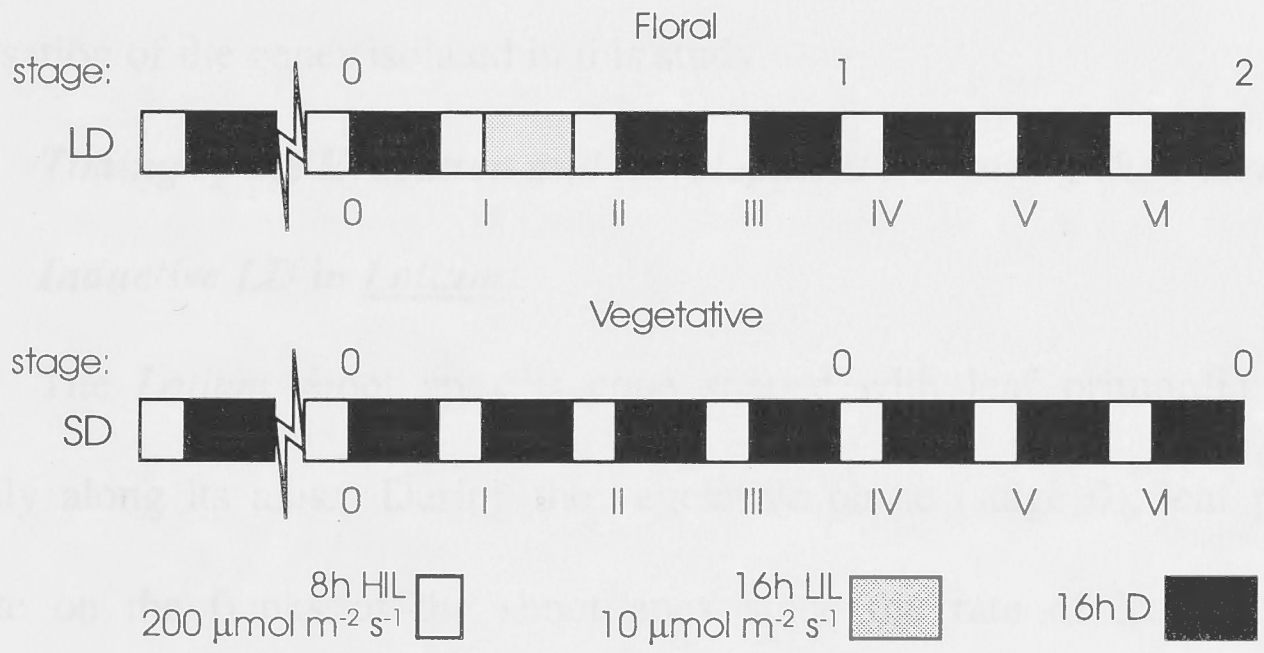


Figure 1.2 Cartoon of SD and inductive LD treatments. The inductive 16 h non-photosynthetic extension of the 8 h SD is indicated with shading with days shown in white and nights in black. Stages of floral development are indicated above. 0 is vegetative, 1 is pre-double ridges and 2 is double ridges. LIL is low intensity light, HIL is high intensity light and D is dark.

Another advantage of the *Lolium* system is the presence of multiple potential spikelet sites on each shoot apex which, because of the internal replication which this offers, is invaluable for *in situ* hybridisation and immunolocalisation experiments. Studies in the grass *Lolium* may have direct application, through molecular synteny, in the regulation of flowering of other Graminaceous species including pasture grasses and cereal crops such as barley, wheat, oats, rye, rice and even corn. Mutants existing in these species may also allow functional characterisation of the genes isolated in this study.

1.2 *Timing of the Evocation and Development Following the Florally Inductive LD in Lolium*

The *Lolium* shoot apex is cone shaped with leaf primordia arranged distichously along its axis. During the vegetative phase (stage 0), leaf primordia accumulate on the flanks of the shoot apex since the rate of leaf primordium initiation (0.65 d^{-1}) is greater than the rate of leaf appearance (0.19 d^{-1}; Evans and Blundell, 1996). At ca. 6 weeks of age, 12 primordia have accumulated with most being no more than crescentic ridges. Graminaceous species such as *Lolium*, barley and maize accumulate leaf primordia during vegetative development, but dicot shoot apices do not.

Many physiological, cellular and morphological changes occur in *Lolium* seedlings induced to flower with a single long photoperiod. A brief increase in the mitotic index was recorded within the apical dome midway though the inductive LD, preceding movement of the floral stimulus out of the leaves and well before commitment of the apex to flower (Jacqmard *et al.*, 1993). On the morning of LD II, transport of the floral inductive stimulus from the leaves to the apex begins, with saturation achieved later that same day (McDaniel *et al.*, 1991). By the morning of

LD III, 100% of shoot apices when excised and cultured *in vitro* develop florally demonstrating that by 48 h after the end of the LD they are committed to flower (McDaniel *et al.*, 1991).

Beginning on LD II the rate of primordium initiation accelerates to 1.66 d^{-1} (Evans and Blundell, 1996). This is the first visible sign for plants induced with a LD. In plants which are old enough to flower in response to it, this increased initiation rate is maintained until the terminal spikelet differentiates at the apical dome, at which time the number of accumulated primordia has nearly doubled to 23. Rapid elongation of the shoot apex occurs generally during the floral transition in most grasses and cereals. By LD IV, cells in the axils of the accumulated leaf primordia begin to divide (stage 1). Development of these axillary primordia is accompanied by other changes within the shoot apex as shown by increased uptake of ^{32}P into nucleic acids and ^{35}S into protein (Rijven and Evans, 1967; Evans and Rijven, 1967). The double ridge stage (stage 2) follows soon after, the doubleness characterised by the axillary buds (spikelet meristems) overtopping leaf primordia. At this stage, the shoot apex is committed to flower.

Over the next few days, the spikelet meristems expand (stage 3) and the leaf primordia begin to regress. Spikelet sites form laterally and terminally. Two sterile glumes (sterile because they do not have florets in their axils) are the first organs to arise on the flanks of each spikelet meristem (stage 4). Their phyllotaxis is distichous like vegetative leaves. Following glume initiation, up to six lemmas (stage 5) are initiated, also with a distichous arrangement, acropetally along the axis of the rachilla (spikelet axis). These are the fertile bracts. A floret meristem (floral meristems) forms soon after in the axil of each lemma (stage 6). Despite not arising

from the floret meristem, each lemma is considered part of the floret (Dahlgren *et al.*, 1985). *Lolium* florets are sessile on the rachilla (see Figure 1.3a).

Therefore, *Lolium* flowers, like those of many grasses consist of a lemma, a palea, three lodicules, three stamens and a carpel primordium (see floral diagram in Figure 1.3b and schematic drawing in Figure 1.3c). The lateral stamens are the first to initiate from the floret meristem (stage 8) closely followed by the anterior stamen and carpel. Posterior to the carpel, the palea forms as a narrow ridge of tissue having two obtuse-shaped tips. Unlike the glumes and lemma which are single keeled (type 1 phytomer), the palea has two keels (type 2 phytomer or prophyll) (Williams, 1975; Bossinger *et al.*, 1992). Last to initiate are the lodicules with one forming posterior to the carpel and two inside the lemma at each side of the base of the anterior stamen. Inside the carpel, the tip of the floret meristem differentiates into a single ovule.

Many aspects of grass floral development, certainly the number of floral organs and its unusual and characteristic mature architecture, differ substantially from that of well known dicot flowers (such as *Arabidopsis*). There are, however, striking similarities. For instance, since the glumes do not have floral meristems in their axils, they are equivalent to cauline leaves in *Arabidopsis* (although cauline leaves have an inflorescence meristem in their axil). Since a floret meristem forms in the axil of each lemma, developmentally the lemma is most similar to a bract. The late initiation and development of the lodicules is strikingly similar to the late development of petals in *Arabidopsis*. Additionally, in the *laxatum-a* mutant of barley, lodicules are transformed to stamens (Bossinger *et al.*, 1992). This mutant suggests the homology of lodicules with petals since a similar conversion of petals to

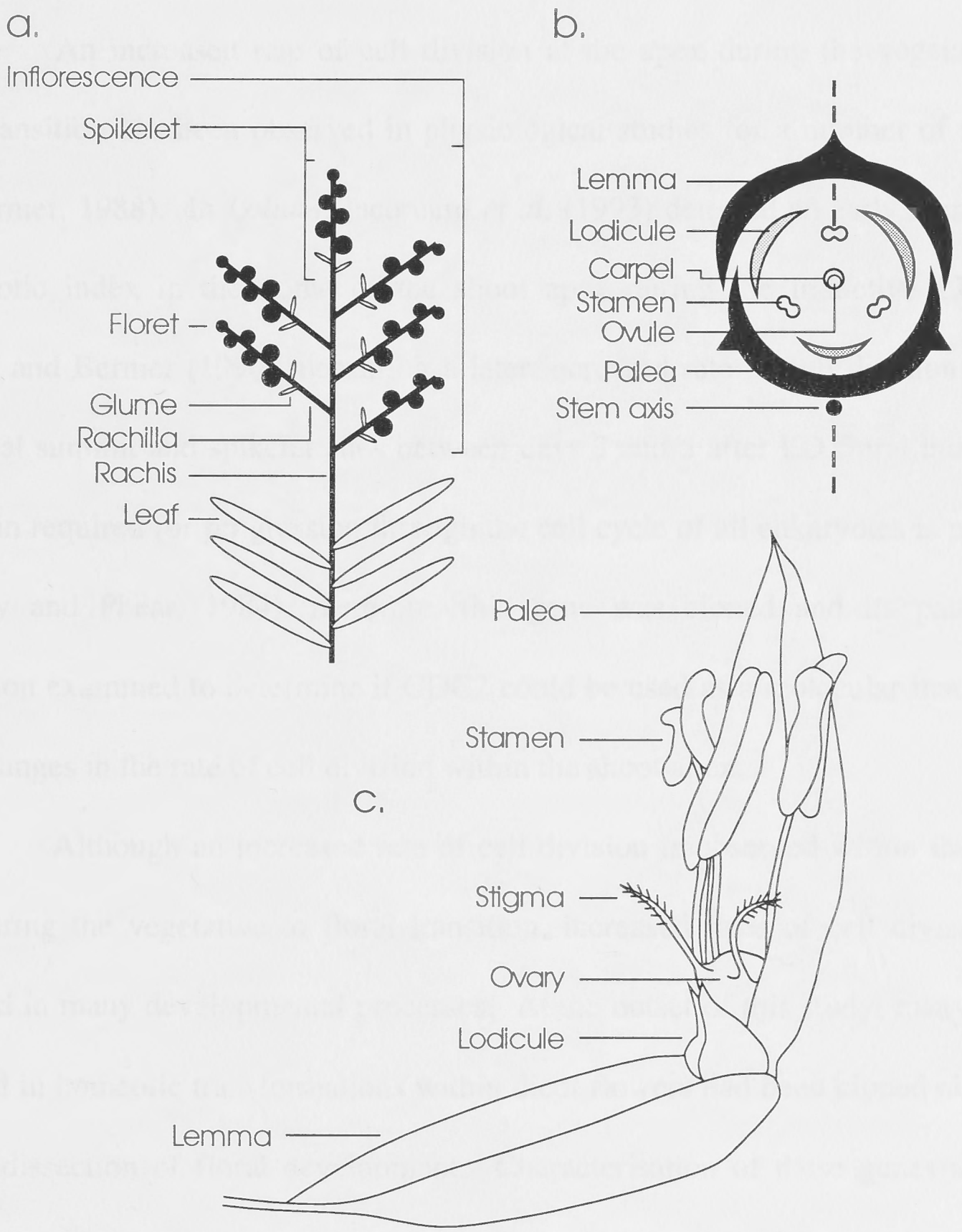


Figure 1.3 Schematic diagram of the above ground morphology a *Lolium* plant in flower (a). *Lolium* floret diagram (b) and schematic drawing of a floret (c).

stamens is observed in the *Arabidopsis apetala2-1* mutant (Meyerowitz *et al.*, 1989). Therefore, the corresponding structures in grass and dicot inflorescences and flowers appear to be glumes/cauline leaves, lemma/bract, palea/sepal, lodicules/petals, stamens and carpels.

1.3 *Basis for Cloning by Homology*

An increased rate of cell division at the apex during the vegetative to floral transition has been observed in physiological studies for a number of species (see Bernier, 1988). In *Lolium*, Jacqmard *et al.* (1993) detected an early increase in the mitotic index in the dome of the shoot apex during the inductive LD with Ormrod and Bernier (1990) measuring a later increased rate of cell division within the apical summit and spikelet sites between days 3 and 5 after LD floral induction. A protein required for progression through the cell cycle of all eukaryotes is $p34^{CDC2}$ (Hindley and Phear, 1984), therefore, this gene was cloned and its pattern of expression examined to determine if CDC2 could be used as a molecular marker for early changes in the rate of cell division within the shoot apex.

Although an increased rate of cell division is observed within the shoot apex during the vegetative to floral transition, increased rates of cell division are observed in many developmental processes. At the outset of this study, many genes involved in homeotic transformations within dicot flowers had been cloned allowing genetic dissection of floral development. Characterisation of these genes and the mutants from which they were cloned led to the elaboration of the ABC model in which A function specifies sepal identity, A together with B function specifies petal identity, B together with C function specifies stamen identity and C function alone specifies carpel identity (Weigel and Meyerowitz, 1994). The genes involved in the ABC model, associated genes involved in meristem identity and genes involved in

determining time to flower all provided a framework which could be used to study the flowering process in a plant species such as *Lolium*. *APETALA1* and *LEAFY*, which specify floral meristem identity in *Arabidopsis*, are expressed early in flower development. Therefore, they might also be expressed early in the in *Lolium* flowering, thus homologues of *APETALA1* and *LEAFY* were cloned and characterised in order to characterise their role during the vegetative to floral transition.

The gibberellin (GA) class of plant growth regulators have long been considered to play a role in flowering (Lang, 1965). *Lolium temulentum* is one LD species which is induced to flower by exogenous application of GA's, even under strictly non-inductive SD's (Evans *et al.*, 1990). Long day-induced flowering in *Lolium* may also be mediated through increases in the level of endogenous GA's (Pharis *et al.*, 1987; McDaniel and Hartnett, 1996). A connection between GA's and flowering is also observed in the LDP *Arabidopsis*. Mutants with very reduced GA levels (e.g., *ga1*-3) are extremely delayed in their flowering under short days (Wilson *et al.*, 1992), whereas, mutants such as *spindly* (*spy*) and *elongated*, having the appearance of being treated with high levels of GA, flower early (Jacobsen and Olszewski, 1993; Halliday *et al.*, 1996). A critical link between GA and gene expression may involve the transcription factor GAMYB which has been demonstrated to be a component of GA-mediated signal transduction in cereal aleurone where it increases α-amylase expression (Gubler *et al.*, 1995). This gene may also transcriptionally activate flowering genes in response to increased GA status. Since there is no evidence to indicate whether GAMYB is a component of the GA signal transduction pathway in other cell types, I aimed to determine if this

gene was expressed in *Lolium* shoot apices and to characterise its role during the vegetative to floral transition.

1.4 *Thesis Objectives*

The aim of this thesis was to examine some of the molecular aspects of evocation, although it is clearly only one aspect of the flowering process. The questions which I seek to answer are:

(i) Are homologues of the well characterised homeotic genes such as *LEAFY* and *APETALA1* in *Arabidopsis* involved in the early stages of floral evocation/development in *Lolium*?

(ii) Is the regulation of the flowering process in *Lolium* similar to that in LDP's such as *Sinapis alba*, *Arabidopsis* and *Antirrhinum*?

(iii) If homologues of "flowering" genes are obtained, how does their localisation differ temporally and spatially in *Lolium* compared to that in dicot systems?

(iv) Does the temporal and spatial localisation of homologous genes in *Lolium*, compared with their localisations in dicot systems, shed light on the evolutionary relationship between the monocot and dicot floral structures?

(v) Is GAMYB expressed within the shoot apex during the vegetative to floral transition in *Lolium*?

(vi) Finally, can the molecular characterisation of known "flowering" genes in *Lolium* be used to identify novel genes involved in the evocation process?

CHAPTER 2

METHODS

Although the apical meristem is responsible for laying down the entire above-ground body of a plant, it is certainly not an abundant source of material for molecular botanical analysis. A vegetative *Lolium* apex is a cone of about 700 µm in length by 150 µm in diameter at its base having an average dry weight of 2 µg. As a result, PCR methods were required for most of the work herein described. The primary focus of this chapter is to detail the specifics of how PCR was used to generate libraries and how the PCR-based northern blots were generated.

Brief sections will provide descriptions of routine molecular techniques as well as the plant growth conditions and the photoperiodic induction treatment. Methods for *in situ* mRNA analysis and immunolocalisation have been modified from existing methods and are detailed in Appendices I and II.

2.1.1 *Plant Material*

Lolium temulentum L. strain Ceres seedlings were grown at the rate of two per 7.5 cm pot in a 1:1 mixture of perlite:vermiculite. This was irrigated with nutrient solution each morning and water each afternoon (Evans, 1969). Prior to treatment, plants were grown for 6 to 7 weeks under 8 h short days (SD's) with a day temperature of 25°C and 16 h dark with a night temperature of 20°C. For the first 5 weeks, growth was under natural daylight in shuttered cabinets to control daylength. Thereafter, the tillers and lower main stem leaves were removed and plants transferred to cabinets with photo- and thermo-periods identical to those above. The cabinets were illuminated with fluorescent and incandescent lamps providing a total

irradiance of 250 to 300 μmol $m^{-2} \cdot s^{-1}$ photosynthetically active radiation (PAR) at plant canopy height with a red to far-red ratio of 2.3.

2.1.2 *Treatment*

Comparisons were made across several stages of floral development and with vegetative SD control plants of equivalent ages. The single inductive LD, designated as LD I, was provided as a 16 h extension of the 8 h SD photoperiod with low irradiance (10 to 12 μmol $m^{-2} \cdot s^{-1}$ PAR) illumination from incandescent lamps.

Shoot apices were harvested at several times following LD floral induction in parallel with vegetative controls. Apices for *in situ* hybridisation or immunolocalisation were fixed in FAA on ice as detailed in Appendix I. Harvests of shoot apices for mRNA extraction were done on dry ice with the tissue subsequently stored at -70°C. Shoot apex tissue included accumulated leaf primordia, but not the leaf primordium enfolding the apex.

In each experiment, the vegetative status of 8 to 10 uninduced controls and the floral status of 8 to 10 LD-treated plants was assessed by dissection at a fixed time three weeks post-induction. Two directly related measures of inflorescence development were employed, floral stage and shoot apex length. The stage of floral development was recorded according to the scale of Evans *et al.* (1990) where: 0 is vegetative; 1 is pre-double ridges with incipient swelling of the spikelet sites; 2 is double ridges which is the first stage at which floral commitment of the shoot apex is externally apparent; 3 is advanced double ridges; 4 is glume primordia; 5 is lemma primordia; 6 is floret primordia; and 8 is anther primordia on the most advanced spikelet. Where inflorescence development was compared over time, apices were assessed at each dissection thereby allowing their rate of development to be

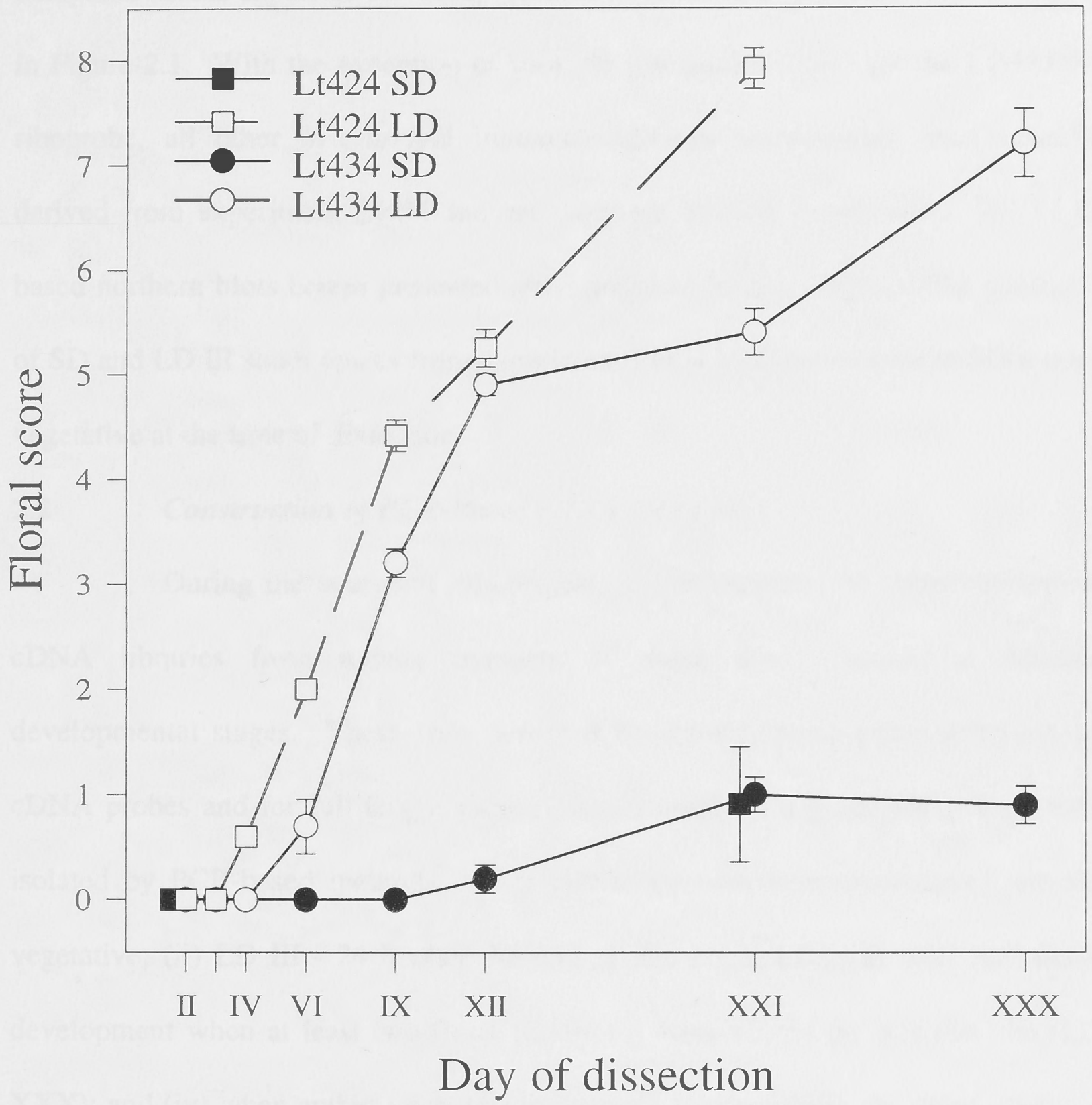


Figure 2.1 Floral score of the shoot apices from experiments Lt424 and Lt434 as a function of time after a single inductive LD.

Floral stage was assessed on several days after LD floral induction using the scale of Evans *et al.* (1990) by dissection of intact apices. Each point is the mean of 10 replicate plants ± S.E.

compared across experiments. A representative developmental timecourse is shown in Figure 2.1. With the exception of some *in situ* results shown for the LtMADS1 riboprobe, all other *in situ* and immunolocalisation experiments used material derived from experiment Lt434 and are therefore directly comparable. All PCR-based northern blots herein presented were prepared from a single cDNA synthesis of SD and LD III shoot apices from experiment Lt424. All plants kept in SD's were vegetative at the time of dissection.

2.2 *Construction of PCR-Based cDNA Libraries*

During the course of this project, it was necessary to construct several cDNA libraries from minute amounts of shoot apex material at specific developmental stages. These were screened for known genes using heterologous cDNA probes and for full length transcripts of homologous genes using fragments isolated by PCR-based methods. Four cDNA libraries were constructed: (i) SD vegetative; (ii) LD III - 24 h after the end of the single LD; (iii) well into floral development when at least two floret meristems were visible per spikelet site (LD XXX); and (iv) when anther primordia were being initiated from the floret sites late in development (LD L) [See overview of library construction method in Figure 2.2.] Although efficient extraction of mRNA and synthesis of PCR-based cDNA from as few as 14 shoot apices is routine, to ensure that each library was representative each was constructed from not less than 5.0 mg F.W. of shoot apex tissue (equivalent to ca. 250 vegetative shoot apices). It has been shown that PCR from a limiting amount of a complex template displays the "Monte Carlo effect." Essentially, if the starting pool is too small, low abundance mRNAs (≤0.04% of the polyadenylated mRNA pool) become inaccurately represented in the PCR-based cDNA. Put another way,

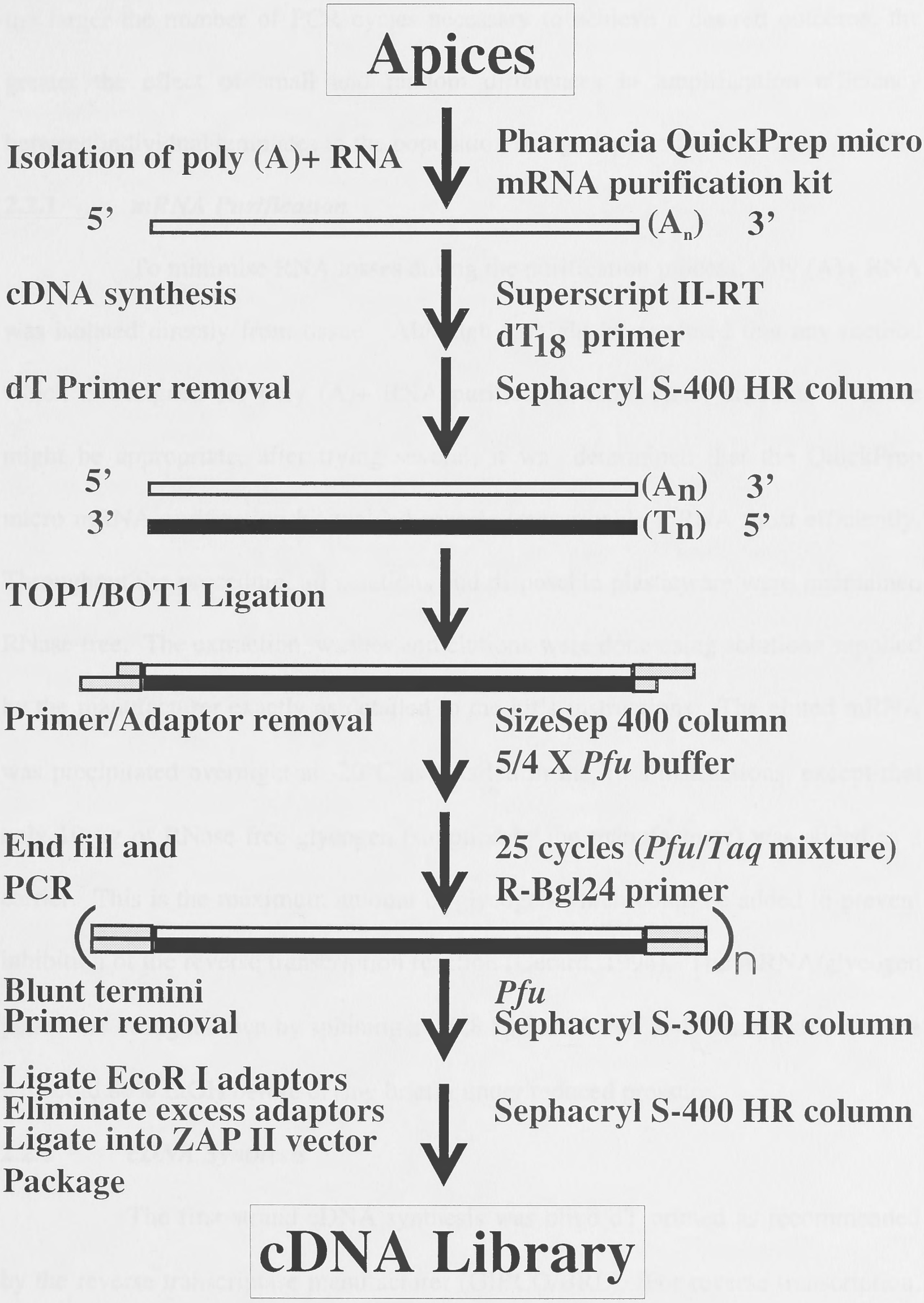


Figure 2.2 Scheme for the construction of PCR-based shoot apex specific cDNA libraries.

the larger the number of PCR cycles necessary to achieve a desired outcome, the greater the effect of small and random differences in amplification efficiency between individual templates in the population being amplified (Karrer *et al.*, 1995).

2.2.1 *mRNA Purification*

To minimise RNA losses during the purification process, poly (A)+ RNA was isolated directly from tissue. Although it might be expected that any method which is designed for poly (A)+ RNA purification from small amounts of tissue might be appropriate, after trying several, it was determined that the QuickPrep micro mRNA purification kit yielded reverse-transcribable mRNA most efficiently. Throughout the procedure, all solutions and disposable plasticware were maintained RNase free. The extraction, washes and elutions were done using solutions supplied by the manufacturer exactly as detailed in the kit's instructions. The eluted mRNA was precipitated overnight at -20°C as detailed in the kit's instructions, except that only 10 µg of RNase free glycogen (supplied by the manufacturer) was added as a carrier. This is the maximum amount of glycogen which could be added to prevent inhibition of the reverse transcription reaction (Gerard, 1994). The mRNA/glycogen pellet was brought down by spinning at 20.8 k*g* for 30 min at 4°C and washed twice with cold 80% EtOH before drying briefly under reduced pressure.

2.2.2 *cDNA Synthesis*

The first strand cDNA synthesis was oligo dT primed as recommended by the reverse transcriptase manufacturer (GIBCO/BRL). For reverse transcription, the mRNA was resuspended in 10 µL of DEPC-H_2O with 1.0 µL Oligo $dT_{12\text{-}18}$ (0.5 µg - NEB) and 1.0 µL RNasin (39 U - Promega) added. Secondary structure was removed from the mRNA by heat denaturation at 70°C for 10 min prior to first

strand synthesis. After chilling the tube on ice and bringing its contents to the bottom, the 20 µL first strand reaction was made to final concentrations of 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT and 500 µM each dNTP. After adding 200 U of Superscript™ II reverse transcriptase (GIBCO/BRL), the reaction was incubated for 10 min at room temperature to assist oligo dT binding followed by 60 min at 42°C to extend the first strand products.

The second strand synthesis reaction was also performed as recommended by GIBCO/BRL. On ice 140 µL of second strand reaction mix [21 mM Tris-HCl, pH 6.9, 103 mM KCl, 5 mM $MgCl_2$, 11 mM $(NH_4)_2SO_4$, 200 µM each dNTP, 4 mM DTT, 170 µM ß-NAD (Boerhinger), 15 U of *Escherichia coli* DNA ligase (NEB), 40 U *E. coli* DNA polymerase I (NEB), 1.4 U of RNase H (Promega)] was added and incubated at 16°C for 2 h. The cDNA were blunt-ended by adding 10 U of T4 DNA polymerase and incubating the reaction at 16°C for 5 min.

To terminate the cDNA synthesis reaction it was extracted once with an equal volume of phenol:[chloroform:IAA] (1:[24:1]) and once with chloroform. Efficient extraction was ensured by vortex mixing the organic and aqueous phases for 30 s.

The extracted cDNA was subsequently precipitated in final concentrations of 0.3 M NaOAc (pH 4.8) and 70% EtOH in the presence of 20 µg of glycogen carrier for at least 1 h at -20°C. The cDNA was pelleted, washed and dried as in section 2.2.1.

2.2.3 *Primer/Adaptor Ligation*

In order to PCR amplify the cDNA, it was necessary to ligate a primer/adaptor to each end of the cDNA. DNA oligomers designated TOP1 [5'd(AGCACTCT-CCAGCCTCTCACCGCAGTC**GATC**GT)-3'] and BOT1 [5' d(pACGATCGAC)-3'] were annealed at equimolar concentration by slow cooling from 50°C to 14°C over 1 h to produce the TOP1/BOT1 double-stranded primer/adaptor with one blunt end and one 24-bp overhang. This primer/adaptor was based on the R-Bgl24/12 set of oligomers published by Lisitsyn *et al.* (1993), but was redesigned to enable ligation to blunt ended cDNA. Additionally, it had a Dpn II restriction site (indicated in bold in the TOP1 oligomer) incorporated close to the site of ligation. This site enabled any PCR-based cDNA to be used in cDNA RDA, a PCR-based subtraction (data not presented herein).

The TOP1/BOT1 primer/adaptor takes advantage of suppression PCR technology, minimising the generation of PCR artefacts common to non-suppression type PCR reactions (see Figure 2.3). With such a primer/adaptor, the termini of the upper and lower strands of each cDNA molecule are complementary and can therefore anneal either intramolecularly or with the added PCR primer. The latter results in exponential amplification of the cDNAs.

To prevent the dT primer from being ligated to the TOP1/BOT1 primer/adaptor, the dried cDNA from above (2.2.2) was resuspended in 50 µL TM buffer [60 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$]. This was applied to a Microspin™ Sephacryl S-400 HR column (Pharmacia Biotech) which had been preconditioned with six 500µL volumes of TM buffer, microfuging for 5 s between each addition. The suspended DNA was then applied to the Microspin™ column

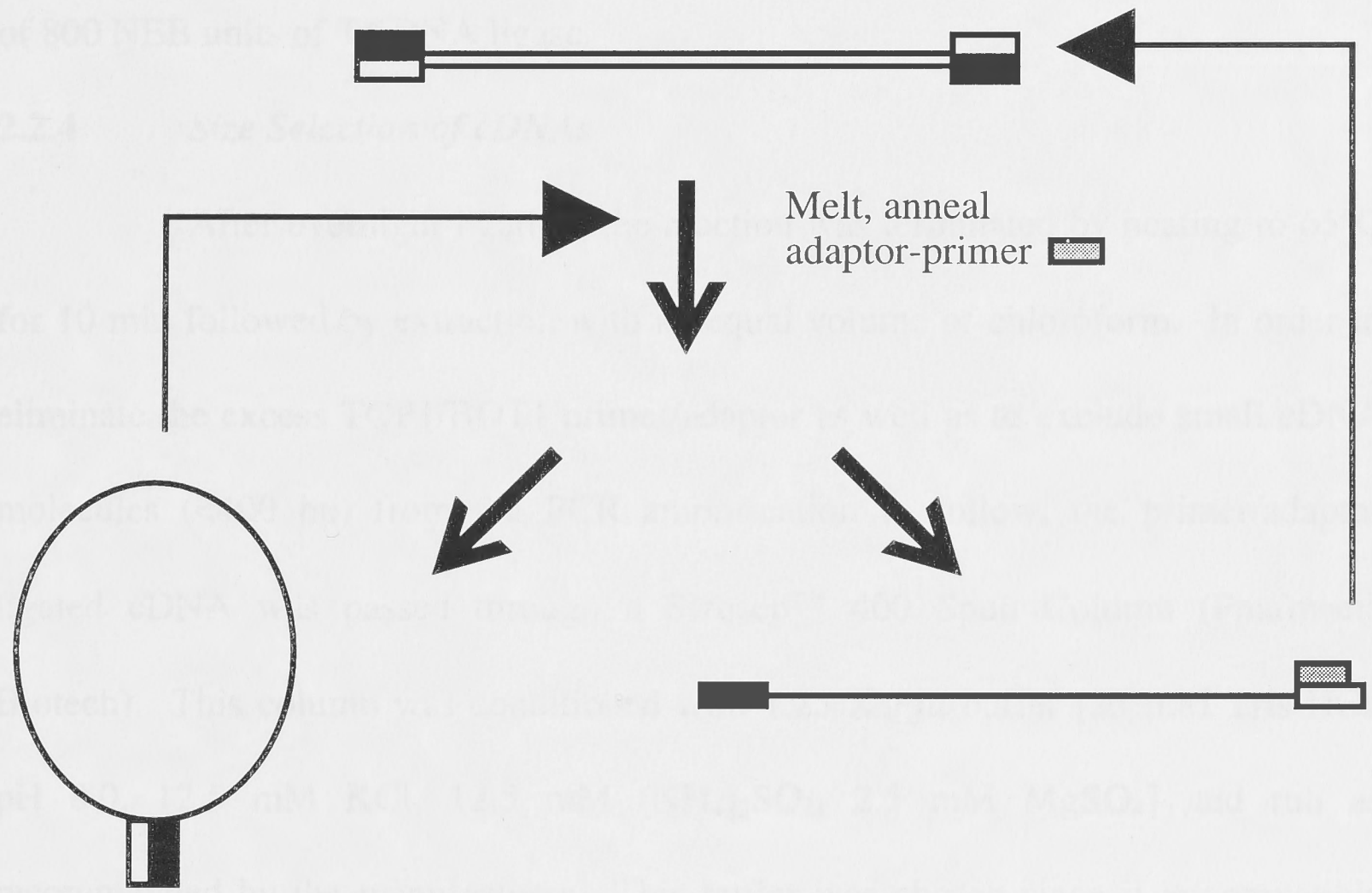


Figure 2.3 Theory of suppression PCR.

The sequences on the ends contain inverted terminal repeats which will form 'panhandle' structures which suppress non-specific PCR amplification (adapted from Seibert *et al.*, 1995).

and eluted as recommended by the manufacturer.

The eluate was then ligated at 10°C overnight in the presence of 250 pmol of annealed TOP1/BOT1 primer/adaptor in T4 ligase/kinase buffer [60 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 10 mM DTT, 1 mM rATP] following the addition of 800 NEB units of T4 DNA ligase.

2.2.4 *Size Selection of cDNAs*

After overnight ligation, the reaction was terminated by heating to 65°C for 10 min followed by extraction with an equal volume of chloroform. In order to eliminate the excess TOP1/BOT1 primer/adaptor as well as to exclude small cDNA molecules (<400 bp) from the PCR amplification to follow, the primer/adaptor ligated cDNA was passed through a Sizesep™ 400 Spun Column (Pharmacia Biotech). This column was conditioned with 1.25 X *Pfu* buffer [25 mM Tris-HCl, pH 8.0, 12.5 mM KCl, 12.5 mM $(NH_4)_2SO_4$, 2.5 mM $MgSO_4$] and run as recommended by the manufacturer. This buffer was chosen since it is compatible with both the spun column as well as with the subsequent PCR procedure. Water was added equal to 1/4 the total volume of the column eluate.

2.2.5 *PCR Amplification of cDNAs and Product Analysis*

The cDNAs were PCR amplified with a *Pfu*:*Taq* DNA polymerase mixture (1:10 on an activity basis), which enabled the synthesis of higher fidelity products than with *Taq* DNA polymerase alone (Cline *et al.*, 1996). Essentially, PCR of a 2 µL aliquot of the eluate from 2.2.4 was performed in a 100 µL reaction containing *Pfu* DNA polymerase buffer [20 mM Tris-HCl, pH 8.75, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton® X-100, 0.1 mg mL^{-1} BSA], 2 µM R-Bgl24 primer [5'd(AGCACTCTCCAGCCTCTCACCGCA)-3'], 200 µM of each

dNTP, 10 U *AmpliTaq* DNA polymerase (Perkin Elmer) and 1 U *Pfu* DNA polymerase (Stratagene). PCR was performed in 200 µL capped thin wall tubes (Advanced Biotechnologies LTD) in a Corbett 9600 plate PCR machine. To enable amplification of the cDNAs, the recessed 3' ends were extended in the first step of the PCR program for 5 min at 68°C resulting in blunt ended primer/adaptors on each end of each cDNA molecule. The next step was a denaturation at 94°C which was followed by 25 cycles of amplification including a 30 s denaturation step at 94°C followed by an annealing/extension step at 68°C for 4 min, with the final cycle having a 10 min extension step.

For each library, four 100 µL PCR reactions were combined and extracted thrice with an equal volume of chloroform and precipitated on ice for 10 min in final concentrations of 0.3 M NaOAc (pH 4.8) and 70% EtOH. The cDNA inserts were then pelleted by spinning for 15 min at 15 k*g* at room temperature, washed and dried as in section 2.2.1.

Uneven 3' termini resulting from the terminal deoxynucleotide transferase activity of *Taq* DNA polymerase were made blunt with *Pfu* DNA polymerase (Costa and Weiner, 1994). The dried PCR-amplified cDNA from above was resuspended to a final volume of 40 µL in *Pfu* DNA polymerase buffer (see section 2.2.5) with 100 µM dNTP and 5 U of *Pfu* DNA polymerase (Stratagene) incubating at 72°C for 30 min. The blunt ending reaction was terminated by extracting twice with an equal volume of phenol:[chloroform:IAA] (1:[24:1]) and once with chloroform (as in section 2.2.2).

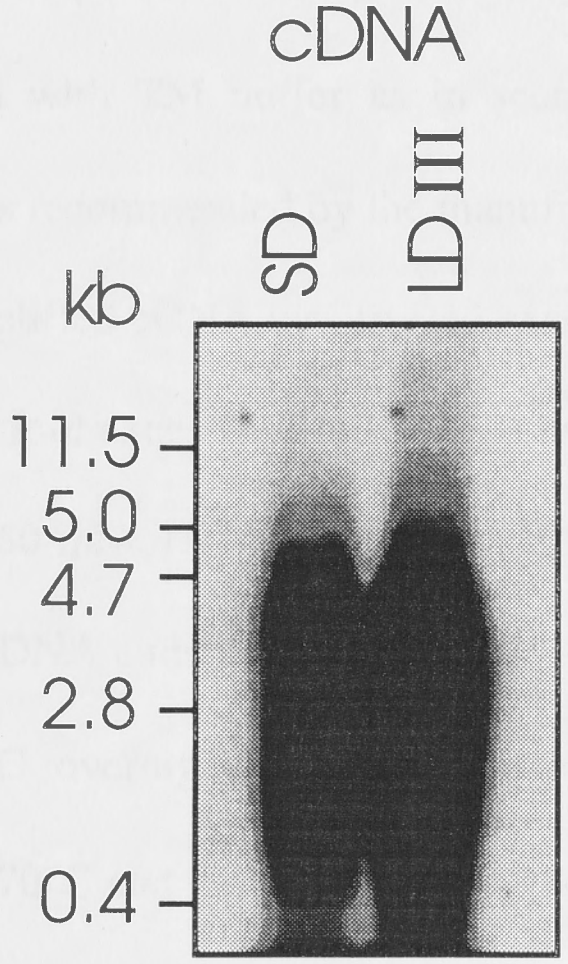


Figure 2.4 Size distribution PCR-amplified cDNA.

PCR-amplified cDNA from SD vegetative or LD III florally induced *Lolium temulentum* strain Ceres shoot apices. An aliquot of TOP1/BOT1-ligated cDNA from 250 shoot apices was PCR-amplified for 25 cycles, electrophoresed on a 1.2% agarose gel and hybridised against itself.

One quarter of the blunt ended cDNAs were analysed by electrophoresis through a 1.2% agarose gel. For each library about 1 µg of cDNA with a size range between 0.4 to 4.5 kbp was observed in this diagnostic gel (see Figure 2.4).

2.2.6 *Linker Ligation and Library Construction*

To eliminate nucleotides and trace phenol, the extracted products were applied to a Microspin™ Sephacryl S-300 HR column (Pharmacia Biotech) which had been preconditioned with TM buffer as in section 2.2.3. The Microspin™ column was then eluted as recommended by the manufacturer.

The PCR-amplified cDNA was treated exactly as that for a conventional library. Essentially, a 10-fold excess of EcoR I adaptors (Stratagene) were ligated in T4 ligase/kinase buffer [60 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 10 mM DTT, 1 mM rATP] to the blunt cDNA ends in a 40µL reaction containing 800 NEB units of T4 DNA ligase at 10°C overnight. The following day, the ligase was heat inactivated for 30 min at 70°C and the 5' end of the 13-mer oligonucleotides kinased in T4 polynucleotide kinase (NEB). Thereafter, the kinase was heat inactivated for 30 min at 70°C then extracted once with phenol:chloroform:isoamylalcohol (v:v:v 25:24:1) and once with chloroform (as per section 2.2.2).

Excess EcoR I adaptors and trace amounts of phenol were eliminated by applying the extracted products to a Microspin™ Sephacryl S-400 HR column (Pharmacia Biotech), preconditioned and eluted exactly as in section 2.2.3. Ligatability of the PCR amplified cDNA was tested with a small aliquot (1 to 5 µL) of the column eluate. Conditions for a large scale ligation into lambda-ZAP® II vector arms were determined by the results of the trial plasmid ligation.

Primary libraries were titered and found to contain between 2 and 4 million phage with the proportion of empty phage being less than 5%, and the average insert size being 1.2 kb. Although each library was constructed from a small amount of tissue, multiple PCR products from the same transcript were never isolated (determined from their 5'-ends). PCR-based errors were eliminated by comparing the sequence for at least three clones over the entire open reading frame (ORF). Of 26 full length clones isolated from the PCR-based libraries, their poly A tails ranged from 4 to 22 nucleotides and their 5'-UTR's ranged from 80 to 140 nucleotides.

2.3 *General Methods*

The many "standard" molecular biology protocols used in this work are detailed in Sambrook *et al.* (1989). Several modified methods were also used. For these, brief summaries are provided with reference to the original source for additional information.

2.3.1 *Generation of Gene Specific Fragments*

Short fragments from known genes were amplified by PCR and cloned into pBluescript SK(+) plasmid vectors for use as specific probes for library screening, RNA or DNA blot analysis. In most cases the primers were designed based on known *Lolium* sequence; however, in rare cases degenerate primers, designed on existing sequences in the database, were made. Gene fragments, in some cases, were amplified from genomic DNA to confirm the sequence and position of introns. Oligonucleotide primers were synthesised on an Applied Biosystems DNA Synthesiser.

In general, 50 µL reactions containing *Taq* DNA polymerase buffer [10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$], 200 µM of each dNTP, 2 µM

of each primer, 50 ng of template DNA and 5 U *AmpliTaq* DNA polymerase (Perkin Elmer) were used to amplify each gene fragment. In various situations, the template consisted of genomic DNA, plasmid DNA or double stranded cDNA (stated in the text). Standard cycling conditions included an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation for 10 s at 94°C, annealing for 30 s at 53°C and extension at 72°C for n/16 s (where n is the length of the expected PCR product). In all cases, the final cycle was extended for 10 min. When degenerate primer pairs were used, touchdown PCR was employed with the denaturation and extension conditions as detailed above. In the touchdown PCR profile, annealing temperatures decreased from T_m+8°C to T_m-8°C in increments of 2°C for eight sets of 5 cycles each, with a final set of 20 cycles performed at the T_m for the primer pair. Melting temperatures (T_ms) were calculated by the OLIGO 4.1 Primer Analysis Software (National Biosciences Inc.).

2.3.2 *Library Screening*

Lolium temulentum L. strain Ceres apex specific PCR-based cDNA libraries (SD, LD III, LD XXX or LD L (see section 2.2 for library construction method)) were plated at a density of 50 000 pfu per 150 mm plate and screened with random primed ^{32}P-dCTP labelled probes (Amersham, Megaprime™ DNA labelling system, RPN 1604; Feinberg and Volgelstein, 1983). Hybridisation was always overnight in 5 X SSPE (1 X SSPE is 150 mM NaCl, 10 mM $NaH_2PO_4 \cdot H_2O$, 1 mM EDTA, pH 7.4) and 0.5% SDS, 5 X Denhardt's solution (0.1% Ficoll, 0.1% PVP, 0.1% BSA) and 25 µg/mL salmon sperm DNA at 65°C. For homologous probes, washes were performed at 65°C, with the first two washes for 30 min in 2 X SSPE and 0.1% SDS and the final wash for 30 min in 0.1 X SSPE and 0.2% SDS. Positive

plaques were purified by a further round or rounds of plaque hybridisation. Excision of pBluescript SK(+) plasmids was performed according to the manufacturer's (Stratagene) instructions.

2.3.3 *DNA Gel Blot Analysis*

Genomic DNA was isolated from two week old etiolated *Lolium temulentum* L. strain Ceres seedlings by the method of Dellaporta *et al.* (1983). In all cases, 20µg of DNA was digested with Bgl II, EcoR I, Hind III or Xho I. The cut DNA was fractionated on a 1% agarose gel and blotted onto Hybond N as per the manufacturer's instructions (Amersham Corp.) (Southern, 1995). Probes were labelled by the random priming method with ^{32}P-dCTP (Feinberg and Vogelstein, 1983; Megaprime kit, Amersham). Hybridisation was in 5 X SSPE, 5 X Denhardt's solution, 0.5% SDS, 25 µg/mL salmon sperm DNA overnight at 65°C. Washes were as indicated in the text.

2.3.4 *PCR-based (Virtual) RNA Blot Analysis*

For all PCR-based (virtual) RNA blots, poly (A)+ RNA was extracted from 250 SD apices (vegetative, stage 0 having an average length of 0.75 mm) or LD III apices (induced, stage 0 having an average length of 0.76 mm) (experiment Lt424; see Figure 2.1). cDNA was synthesised, ligated to TOP1/BOT1 annealed primer adaptors, size selected and PCR-amplified as described in sections 2.2.1 to 2.2.5. (Apices from this experiment reached double ridge stage at LD VI.) The PCR-amplified cDNA from three parallel reactions was combined, chloroform extracted twice, precipitated and microfuged as described in section 2.2.5. After washing, each combined sample was resuspended in 65 µL of 10 mM Tris pH 7.6, 1 mM EDTA and 10 µL of each of the SD and LD III pools loaded in parallel on six 1.2%

agarose gels which were electrophoresed, blotted and hybridised as detailed in section 2.3.3. Washes were as indicated in the text. Hybridisations with virtual RNA blots produced from different amplifications using the same probe always gave identical results. For each set of blots, a fragment of a *Lolium* tubulin gene was used to compare RNA loading across lanes.

2.3.5 *RNA Gel Blot Analysis*

RNA was isolated from *Lolium temulentum* L. de-embryonated half seeds by the method of Schuurink *et al.* (1996). Leaf and root RNA were isolated by the hot phenol method of Chandler *et al.* (1983). Essentially, 2 g of frozen tissue was ground to a fine powder in liquid N_2. The powdered tissue was then added to 9 mL of hot (65°C) phenol:chloroform:IAA (25:24:1) and vortexed for 1 minute. To this was added an equal volume of RNA extraction buffer (100 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1% SDS) and the mixture vortexed again. In order to separate the phases, the mixture was centrifuged for 10 min at 10 k*g*. The aqueous phase was removed to a fresh tube and precipitated with 0.1 volumes 3 M NaOAc (pH 4.8) and 2.5 volumes EtOH at -20°C for 1h and centrifuged for 30 min at 10 k*g*. After centrifugation the pellet was washed with cold 80% EtOH and resuspended in 60 µL RNAse-free water. The resuspended RNA was reprecipitated overnight at -20 °C with 20 µL 8 M LiCl, centrifuged for 20 min at 4°C at 14 k*g*, washed with cold 80% EtOH, dried and resuspended.

For RNA gel blot analysis, 20 µg of total RNA was size fractionated by gel electrophoresis under denaturing conditions in a 1% agarose gel with formaldehyde, transferred to a Hybond-N membrane (Amersham Corp.), and hybridised exactly as in section 2.2.3. Following autoradiography, RNA gel blots

were stripped and reprobed with a 9 kb wheat rRNA clone pTA71 (Gerlach and Bedbrook, 1979), to compare RNA loading across lanes.

2.3.6 *Sequencing and Analysis*

Cloned DNA fragments were sequenced on both strands by the method of Sanger *et al.* (1977) with either Dye Primer or DyeDeoxy Terminators using an ABI PRISM 377 sequencer (Foster City, CA).

Sequence analysis was done using the University of Wisconsin GCG (Genetics Computer Group, Version 8, Madison, Wisconsin, USA) software package.

2.3.7 *Protocol for Rapid Amplification of cDNA Ends (RACE)*

The Seibert *et al.* (1995) method, used to walk to uncloned genomic DNA, was modified to allow the cloning of missing 5'-ends. A 3'-blocked primer/adaptor oligonucleotide was ligated to both ends of a blunt double stranded cDNA template using the method described in section 2.2.3. This cDNA consisted of either unamplified second strand cDNA (generated as detailed in sections 2.2.1 to 2.2.2) or PCR-based cDNA (generated as detailed in section 2.2.1 to 2.2.5). The amine-blocked primer/adaptor was constructed by annealing TOP2 [5'd(CTAATAC-GACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT)-3'] and BOT2 [5' d(pACCTGCCC-NH_2)-3'] oligonucleotides at equimolar concentration (Seibert *et al.*, 1995).

For each 5'-RACE experiment, a pair of nested primers (each 18 to 21 nucleotides in length) complementary to known nucleotide sequence within ca. 200 nucleotides of the existing 5'-end were selected using the OLIGO 4.1 Primer Analysis Software package (National Biosciences Inc.).

Three rounds of PCR were performed in *Pfu* buffer with primer and nucleotide concentrations as specified in section 2.2.5. Adaptor primer 1 (AP1) and a gene specific primer were used to amplify in the first round and AP2 and a nested gene specific primer used in the second and third rounds (Seibert *et al.*, 1995). The *Taq*/*Pfu* mixture specified in section 2.2.5 was used in the first two rounds, with *Pfu* alone used in the final round to improve cloning efficiency (Costa and Weiner, 1994). Standard cycling conditions were used in each round, including denaturation at 94°C for 5 min in the first cycle, followed by 35 cycles (25 cycles or 20 cycles respectively in rounds two and three) of denaturation for 10 s at 94°C, annealing for 30 s at 53°C, extension at 68°C for n/5 s (where n is the length of the expected PCR product), and a final extension of 10 min. From each round, the PCR reaction products were chloroform extracted, precipitated, washed and dried as detailed in section 2.2.5. These products were resuspended and analysed by electrophoresis in a 1.2% agarose gel. In all cases, an obvious band was obtained from each round. This was gel purified (Qiaex) and 1/20 of the gel purified product used in the next round. In the final round, the gel purified product was blunt end cloned into the EcoR V site of pBluescript SK(+) plasmid vector.

CHAPTER 3

EARLY INCREASES IN CDC2 EXPRESSION DURING FLORAL EVOCATION OF *LOLIUM*.

Introduction

An increased rate of cell division at the apex during the vegetative to floral transition has been observed in physiological studies for a number of species (see Bernier, 1988). For instance during the first 2 days following LD floral induction of *Silene*, a 29% decrease in cell doubling time is observed (Taylor and Francis, 1989). In *Sinapis* and *Anagallis* the mitotic index (a reliable indicator of an increased rate of cell division) increases considerably in the shoot apical meristem during evocation (refs. in Bernier *et al.*, 1981). Cell cycle changes also occur in *Lolium* soon after long day floral induction. By scanning microdensitometry, Jacqmard *et al.* (1993) detected a 5-fold increase in the mitotic index in the dome of the apex during the inductive LD with Ormrod and Bernier (1990) measuring increases in the rate of cell division within the apical summit and spikelet sites between days 3 and 5 after LD floral induction.

The eukaryotic cell cycle has four phases which in sequence are: G_1 (before DNA replication), S (DNA replication), G_2 (after DNA replication) and M (mitosis). An additional phase termed G_0 is associated with non-proliferative or quiescent cells. A protein required for progression through the eukaryotic cell cycle is a 34-kD serine/threonine kinase encoded by the $p34^{CDC2}$ gene (hereafter referred to as *CDC2*, CDC being short for **C**ell **D**ivision **C**ycle; Hindley and Phear, 1984). This gene was first identified in two yeast species and found to be required for passage through control points existing at the G_1/S and G_2/M boundaries (Nurse and Bisset,

1981; Nurse, 1990). This protein is a catalytic subunit, which complexes with regulatory subunits, the cyclins, that together, are required to traverse these cell cycle control points (Nurse, 1994). Therefore, CDC2 is one member of the **c**yclin-**d**ependant family of protein **k**inases (CDK's).

From various plant species, at least three distinct classes of CDK's have been cloned (Colasanti *et al.*, 1991; Feiler and Jacobs, 1991; Ferreira *et al.*, 1991; Hirayama *et al.*, 1991; Hirt *et al.*, 1991,1993; Bergounioux *et al.*, 1992; Hashimoto *et al.*, 1992; Miao *et al.*, 1993; Fobert *et al.*, 1994, 1996; Segers *et al.*, 1996; Setiady *et al.*, 1996) as well as a number of cyclins (Hata *et al.*, 1991; Hirt *et al.*, 1992; Hemerly *et al.*, 1992; Day and Reddy, 1994; Ferreira *et al.*, 1994a; 1994b; Fobert *et al.*, 1994; Renaudin *et al.*, 1994; Meskiene *et al.*, 1995; Szarka *et al.*, 1995). In addition to regulation of CDC2 activity through complexing with various cyclins, CDC2 kinase activity is regulated by phosphorylation, at residues Thr14, Tyr15 and Thr161 or Thr167 all of which are conserved in yeast, animals and plants (Lewin, 1990; Shaul *et al.*, 1996).

This study, using CDC2 expression as a measure of potential for cell division sought to determine: (i) whether increased CDC2 expression could be detected within the shoot apex following LD floral induction, and (ii) whether increased CDC2 expression was indicative of general activation of the apex, or whether it was confined to regions of the apex where increased DNA replication had been recorded previously (Ormrod and Bernier, 1990).

Results

3.1 *Cloning of a CDC2 Homologue from Lolium*

A 280 bp PCR fragment derived from wheat CDC2 (kindly provided by Dr Pete John, RSBS, The Australian National University, Canberra, A.C.T., Australia) was used to probe a PCR-based northern blot of cDNA from vegetative (SD) and florally induced (LD III) apices. This blot revealed that the level of CDC2 mRNA species detected by this probe increased in florally induced apices within 24 h of the end of the long day (See Figure 3.1).

An aliquot of 360 000 clones from a florally induced LD III PCR-based shoot apex library was screened at high stringency (0.1 X SSPE; 0.2% SDS at 65°C) yielding 11 hybridising plaques. Clones of appropriate size (ca. 1.5 kb) were end-sequenced revealing that five clones were derived from bonafide CDC2 mRNA's. Complete sequencing of these CDC2 clones revealed none was full length at its 5' end. Although all clones were derived from the same gene, they fell into three classes, two clones appeared to be properly spliced, another two clones contained an insertion of 206 bp (nucleotides 490 to 696 in Figure 3.2) and one clone had a deletion of 91 bp (nucleotides 316 to 407 in Figure 3.2). The insertion of 206 bp (nucleotides 490 to 696 of Figure 3.2) apparently represents an unspliced intron since it has conserved +1G and +2 U at the 5' exon/intron boundary (/GU), a conserved -3C, -2A and -1G at its 3' intron/exon boundary (CAG/) a uridine tract near the 3' intron/exon boundary and has an AU content of 62% (Goodall *et al.*, 1991). This AU content is close to the average (56%) for monocot introns (Goodall and Filipowicz, 1989). Although the deletion was only 91 nucleotides (316 to 407 in Figure 3.2), it may have been recognised as an intron since it has a conserved 5'

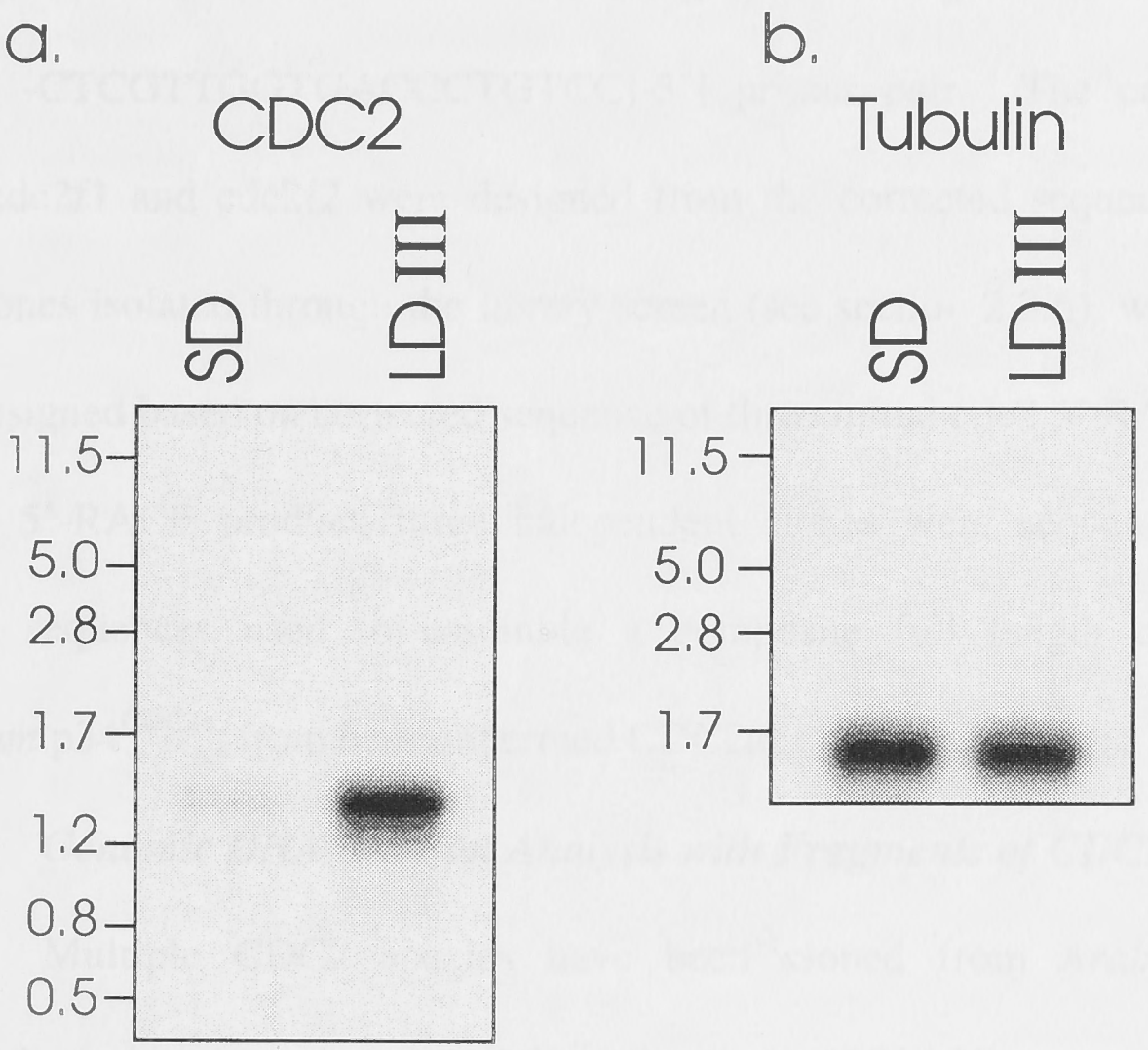


Figure 3.1 Virtual northern blot analysis of vegetative SD and florally induced LD III (24 h after the end of the single LD) PCR-based cDNA hybridised with (a) a fragment of the wheat CDC2a cDNA (b) a fragment of a *Lolium* tubulin cDNA (see Table 2.3.4) to compare loading between lanes. Sizes in kbp are indicated to the left.

exon/intron boundary (/GU), a partially conserved 3' intron/exon boundary (AG/), a short U tract near the 3' intron/exon boundary and an AU content of 61%.

In order to clone the 5'-terminus of this gene, **r**apid **a**mplification of **c**DNA **e**nds (RACE) was performed initially with the nested primers (cdc2f1 [5'-d(G -AGTCCATCAACTTCTTGAG)-3'] and cdc2f2 [5'-d(CATCGTGTAGCTTGACG-ATG)-3'], with the most 5' nucleotides being cloned using the cdc2f2 and cdc2f3 [5'-d(GT -CTCGTTGGTGACCCTGTCC)-3'] primer pair. The cdc2 5'-RACE primers cdc2f1 and cdc2f2 were designed from the corrected sequence of *Lolium* CDC2 clones isolated through the library screen (see section 2.3.6), with the primer cdc2f3 designed based on corrected sequence of three initial cdc2 5' RACE products. For each 5'-RACE product, three independent clones were sequenced and their corrected sequences used to assemble a composite full length clone, *Lolium temulentum* $p34^{CDC2a}$, from here on termed CDC2aLt (shown in Figure 3.2).

3.2 *Genomic DNA Gel Blot Analysis with Fragments of CDC2aLt.*

Multiple CDC2 species have been cloned from *Arabidopsis*, rice, soybean, *Antirrhinum* and corn (Colasanti *et al.*, 1991; Hirayama *et al.*, 1991; Hashimoto *et al.*, 1992; Miao *et al.*, 1993; Fobert *et al.*, 1996), therefore multiple CDC2 species were also expected to exist in *Lolium*. A conserved Hind III site exists in the CDC2a species from at least 9 plant species including corn (CDC2ZmA), rice (CDC2Os-1), *Arabidopsis* (CDC2aAt) and *Lolium* (see Hind III in Figure 3.2). The 3'-end of the CDC2aLt cDNA clone was thus cloned from this Hind III site through to the end of the cDNA and was used to probe a genomic DNA blot. At high stringency (0.1 X SSPE; 0.2% SDS at 65°C), this probe detected multiple hybridising bands (up to 7) within the *Lolium* genome (see Figure 3.3a).

```
   1  ATGGAGCAGTACGAGAAGGAGGAGAAGATCGGGGAGGGCACGTACGGGGTGGTCTACAGG    60
   1  M  E  Q  Y  E  K  E  E  K  I  G  E  G  T  Y  G  V  V  Y  R     20

  61  GCGCGGGACAGGGTCACCAACGAGACCATCGCGCTCAAGAAGATCCGCCTCGAGCAGGAG   120
  21  A  R  D  R  V  T  N  E  T  I  A  L  K  K  I  R  L  E  Q  E     40

 121  GATGAGGGCGTCCCCTCCACCGCCATCCGCGAGATCTCGCTGCTCAAGGAGATGCAGCAC   180
  41  D  E  G  V  P  S  T  A  I  R  E  I  S  L  L  K  E  M  Q  H     60

 181  GGCAACATCGTCAAGCTACACGATGTCATCCACAGCGAGAAGCGCATATGGCTCGTCTTT   240
  61  G  N  I  V  K  L  H  D  V  I  H  S  E  K  R  I  W  L  V  F     80

 241  GAGTACCTGGATCTGGACCTCAAGAAGTTCATGGACTCTTCTCCAGAGTTTGCCAAGAGC   300
  81  E  Y  L  D  L  D  L  K  K  F  M  D  S  S  P  E  F  A  K  S    100
                  ∇
 301  CCTGCTTTGATCAAGTCATATCTCTATCAGATACTCCGCGGCGTTGCTTATTGCCATTCT   360
 101  P  A  L  I  K  S  Y  L  Y  Q  I  L  R  G  V  A  Y  C  H  S    120
 101  P  A  L  I  K                                                 105
                                                        ∇
 361  CATAGGGTTCTTCATCGAGATTTGAAACCTCAGAATTTATTGATAGATCGGCGTACTAAC   420
 121  H  R  V  L  H  R  D  L  K  P  Q  N  L  L  I  D  R  R  T  N    140
 106                                                  I  G  V  L  T    110
        ▼Hind III
 421  GCACTGAAGCTTGCAGACTTTGGTTTAGCCAGGGCATTTGGAATCCCTGTTCGTACATTT   480
 141  A  L  K  L  A  D  F  G  L  A  R  A  F  G  I  P  V  R  T  F    160
 111   H  *                                                         111
           ∇ cdc2i1
 481  ACTCATGAGGTATTTTTCTTCTATCACTCGTTTACAAGTGCCTTTGAGAACTGTTCCAGT   540
 161  T  H  E                                                      163

 541  ACCACGTCGCCTTTTGGCAGGATCTCAGTATTACAATTTTGCATCTTAGTATGTACTACA   600

 601  TATGATTTTGCATCTAAACAAGCTCATTGTTCTTCTATCCATCCAACACCATGACACATC   660
                  cdc2i2                  ∇
 661  AACTCACAAGGCATGTCTATGGGAAGTATTTTTCAGGTAGTAACATTATGGTACAGAGCT   720
 164                                      V  V  T  L  W  Y  R  A     171

 721  CCAGAAATTCTTCTTGGAGCGAGGCAGTATTCCACACCAGTTGATGTGTGGTCAGTGGGC   780
 172  P  E  I  L  L  G  A  R  Q  Y  S  T  P  V  D  V  W  S  V  G    191
                                          cdc2a1
 781  TGTATCTTTGCAGAAATGGTGAACCAGAAGCCACTGTTTCCTGGCGATTCTGAGATTGAT   840
 192  C  I  F  A  E  M  V  N  Q  K  P  L  F  P  G  D  S  E  I  D    211

 841  GAATTATTTAAGATATTCAGGGTACTTGGCACTCCAACAGAACAAACTTGGCCAGGAGTG   900
 212  E  L  F  K  I  F  R  V  L  G  T  P  T  E  Q  T  W  P  G  V    231

 901  AGCTCCTTGCCTGACTATAAGTCTGCTTTCCCCAAGTGGCAGGCAGAGGAGCTGGCCACT   960
 232  S  S  L  P  D  Y  K  S  A  F  P  K  W  Q  A  E  E  L  A  T    251

 961  GTTGTCCCCAATCTTGGATCTGTTGGCTTGGACCTTCTCTCCAAAATGCTTCGGTTCGAG  1020
 252  V  V  P  N  L  G  S  V  G  L  D  L  L  S  K  M  L  R  F  E    271

1021  CCAAGCAAAAGGATCACAGCTAGGCAGGCTTTGGAGCACGAGTACTTCAAGGACATCGAG  1080
 272  P  S  K  R  I  T  A  R  Q  A  L  E  H  E  Y  F  K  D  I  E    291

1081  GTGGTACAATGAGCTGGCTATTTATTTGATGTTGAATTGGTTGGCATATGTATGAGCTGG  1140
 292  V  V  Q  *                                                    294
                                cdc2a2
1141  GCTGCTCATTTCATTCCTTTGTGAACGTTGTGCCTTTGGCATTTTTGTCATTCAGCTGGA  1200
1201  TATTTCGGATCTGGTGTGTTGGGAGGTGTACTCAGGAATGCTAAATAGATTACCATCTTG  1260
1261  GTCTCAAAAAAAAAAAAAA                                           1279
```

Figure 3.2 Nucleotide and predicted amino acid sequence of CDC2aLt cDNA. Numerals to the right of each sequence indicate nucleotide and amino acid positions with the initiation codon providing the start. The second translation line shows the putative translated protein from the cDNA which contains the 91 nucleotide deletion from nucleotides 316 to 407. Stop codons are indicated with an asterisk. Intron-specific (nucleotides 490 to 696) primers cdc2i1 and cdc2i2 and 3'-gene specific primers cdc2a1 and cdc2a2 are indicated above the nucleotide sequence. Amino acid motifs conserved in all CDC2 proteins are underlined. Phosphorylation sites conserved in all CDC2 proteins are indicated in bold. The Hind III restriction site conserved in many CDC2a homologues is indicated with an arrowhead.

Additionally, a 3' end-specific probe for CDC2aLt was generated by PCR (between the primers cdc2a1 and cdc2a2 indicated on Figure 3.2). This 367 bp fragment including a portion of the 3'-UTR was blunt-end cloned into the EcoR V site of pBluescript SK(+). To determine whether it specifically detected the *CDC2aLt* gene, a blot of *Lolium* genomic DNA was probed with this fragment. A single band was detected in each of the four lanes for four different DNA restriction enzyme digests. These results indicate that a single copy gene is recognised by this 3'-end specific probe (see Figure 3.3b).

Given that two of the five CDC2aLt clones isolated from the PCR-based library contained an intron, a probe specific to the 206 bp insertion was PCR-amplified using cdc2i1 [5'-d(ATACTTCCCATAGACAT)-3'] and cdc2i2 [5'-d(ATGAGGTATTTTTCTTC)-3'] primers, in order to determine if both mRNA types originated from transcription from the same gene. When the 206 bp probe was hybridised to a genomic DNA blot, multiple bands were detected (See Figure 3.3c). The strongest hybridising band in each lane probed with either the intron-specific probe or with the *CDC2aLt* fragment 3' of the conserved Hind III site migrated at the same mobility as the single hybridising band in each lane when probed with the 3'-end specific probe (compare Figures 3.3a and 3.3c with Figure 3.3b). Equal mobility of the strongest hybridising band in Figure 3.3c (cdc2i1/2 intron-probed blot) with the bands in Figure 3.3b (3'-end specific-probed blot) suggest that both fragments are derived from the same gene. What is most surprising, is that the sequence of this putative intron is strongly conserved since at high stringency it hybridises with multiple bands on a genomic DNA blot.

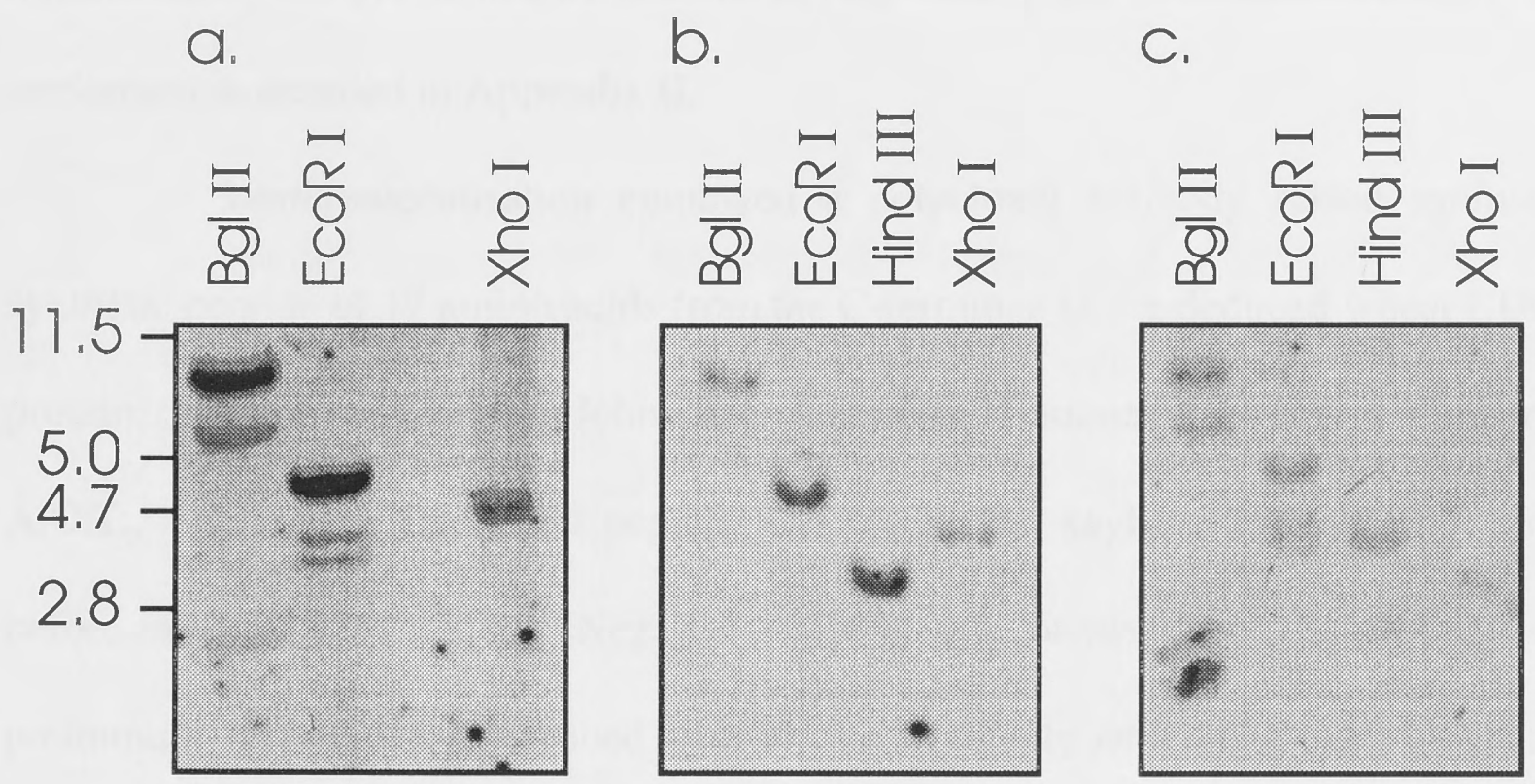


Figure 3.3 *Lolium* genomic DNA gel blot analysis using three homologous probes from the CDC2aLt cDNA.

Genomic DNA (20 μg) was digested with Bgl II, EcoR I, Hind III and Xho I. After blotting, the digested DNA was hybridised with: (a) the 3' end of *CDC2aLt* from the conserved Hind III site through the end of the cDNA (nucleotide 427 to 1279), (b) a PCR fragment (cdc2i1/2) within the intron (nucleotide 485 to 689) or (c) a 3'-*CDC2aLt* gene specific probe (cdc2a1/2; nucleotide 812 to 1179). Sizes in kbp are indicated to the left.

3.3 *CDC2 Expression*

The spatial localisation of CDC2 protein and mRNA was analysed by immunolocalisation and *in situ* mRNA hybridisation, respectively, in sections of *Lolium* shoot apices ranging from vegetative through to LD XXX. *In situ* hybridisation was performed as detailed in Appendix I and immunolocalisation was performed as detailed in Appendix II.

Immunolocalisation employed a polyclonal antibody raised against a synthetic peptide of 17 amino acids from the C-terminus of the deduced wheat CDC2 protein (a gift from Dr Pete John, The Australian National University, Canberra, A.C.T., Australia). The wheat peptide was coupled to keyhole limpet hemocyanin before injection into rabbits. Negative controls were of two types, (i) probed with preimmune serum, and (ii) probed with blocked antibody which had been incubated with 0.17 mg mL^{-1} of the 17 amino acid synthetic peptide for 2 h prior to incubation it with the sections. Detection was with an anti-rabbit secondary antibody conjugated to alkaline phosphatase. The *Lolium* sequence shows strong homology with this wheat peptide (see Figure 3.4) and a single band was evident on a western blot for protein extracted from leaf and root tissue of *Lolium* (data not shown). Extraction of sufficient protein from *Lolium* apices is unrealistic due to their minute size; however, the negative control, in which antibody was competed with the wheat 17 amino acid synthetic peptide, tests its specificity.

For *in situ* mRNA analysis, sense and antisense DIG-labelled riboprobes were synthesised gene specifically for *CDC2aLt* using the 394 bp 3'-end specific fragment which only detected a single band on a genomic DNA gel blot (see Figure 3.3b). Template for the synthesis of the sense riboprobe was produced by recloning the

Wheat sequence: CALEHEYFKDLDVSS

+**********++*++

Lolium sequence: QALEHEYFKDIEVVQ

* = identical amino acid

+ = conservative substitution

Figure 3.4 Comparison of the 17 most C-terminal amino acids of wheat and *Lolium* CDC2 proteins.

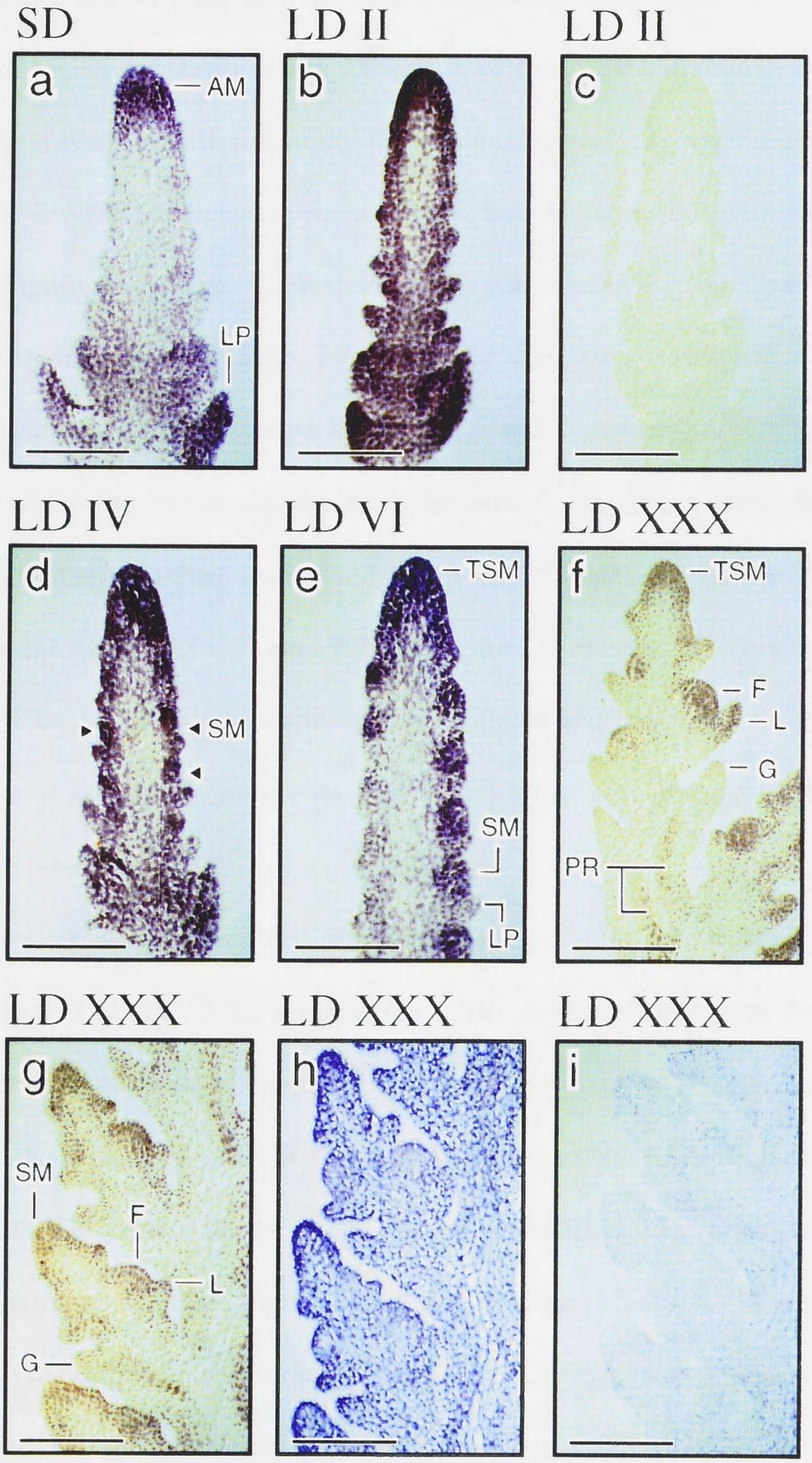
PCR-generated 3'-specific fragment as an EcoR I/Hind III fragment (which flanks the EcoR V site) from pBluescript SK(+) into pBluescript KS(+) in which the multiple cloning site is reversed. Colorimetric detection of the hybridising probes was via an anti-DIG Fab fragment conjugated to alkaline phosphatase.

Over the time range analysed, both *in situ* mRNA localisation for CDC2aLt transcripts and immunolocalisation of CDC2aLt protein with anti-wheat $p34^{cdc2}$ antibody produced similar results. Neither the CDC2aLt sense control (see LD II apex in Figure 3.5c), nor preimmune serum produced detectable staining (data not shown). Staining in vegetative SD shoot apices was concentrated in the apical dome (about 4 cell layers deep) as well as within the leaf primordia at its base (see SD apex in Figure 3.5a). At 2 pm on LD II, the day after the LD, staining within the apex intensified. At this time, CDC2aLt transcript was detected in more cell layers within the apical dome (about 6 cell layers), with new staining being observed along the sides of the apex (about 3 cell layers deep) (see LD II apex in Figure 3.5b). Staining of the leaf primordia at the base of the apex continued to be detected at LD II. By LD IV, 48 h after the end of the LD, staining was strongest in the apical dome and around the edge of the apex. In the LD IV apex seen in Figure 3.5d, the sites of the future spikelet meristems (indicated by arrowheads) can be seen initiating in the axils of the leaf primordia (which continue to stain). Expression of CDC2aLt in the double ridge apex (LD VI to IX) remained concentrated and high in the apical dome as well as within the spikelet meristems, seen as circular zones of cells on the edge of the shoot apex (see LD VI apex in Figure 3.5e). At this stage, little staining remained in the regressing leaf primordia (positioned just below the spikelet meristems; see LD VI apex in Figure 3.5d). At the advanced double ridge stage (LD

Figure 3.5 *In* situ localisation of CDC2aLt transcripts and immunolocalisation of CDC2aLt protein in *Lolium* shoot apices.

In situ hybridisations were performed on longitudinal sections of wax embedded apices at different developmental stages using DIG-labelled *in vitro*-transcribed riboprobes synthesised from the cdc2a1/2 CDC2aLt gene-specific fragment (indicated in Figure 3.2). All sectioned shoot apices were from experiment Lt434 (see Figure 2.1). Sections (a), (b), (d), (e), (f) and (g) were hybridised with the antisense riboprobe. A representative section hybridised with the sense control riboprobe is shown in (c). Hybridisations were with an equivalent sense or antisense probe concentration. The same dilution of anti-DIG antibody was used to detect riboprobe hybridisation and the duration of the colour development was identical for sections (a) through (g). Treatment of sections (h) and (i) was comparable, with section (h) incubated with anti-wheat CDC2 antibody and section (i) with antibody pre-competed for 2 h with the 17 amino acid synthetic peptide. Staining was with a secondary goat anti-rabbit affinity-purified antibody conjugated to alkaline phosphatase (GIBCO-BRL). Signal is visible as the pink to purple product of the alkaline phosphatase reaction. Photographs were taken using Nomarski optics with identical exposures. The staining intensity of sections hybridised with the antisense riboprobe, is directly proportional to the expression of CDC2aLt. Median sections through (a) six-week old vegetative (SD) and (b,c) LD II shoot apices 10 h after export of the floral stimulus from the leaves, (d) LD IV pre-double ridge apex, (e) double ridge apex with five spikelet meristems on right side of the shoot apex (LD VI), (f) terminal spikelet site subtended by two glume primordia with floret meristems alternately along the spikelet axis subtended by lemma primordia (LD XXX), (g, h) two and (h) three lateral spikelet sites at floret stage (LD XXX). The bars are 50 μm.

Abbreviations: AM, apical meristem; F, floret; G, glume; L, lemma; LP, leaf primordium; P, provascular strand; SM, spikelet meristem; TSM, terminal spikelet meristem.


SD
a
AM
LP
LD II
b
LD II
c
LD IV
d
SM
LD VI
e
TSM
SM
LP
LD XXX
f
TSM
F
L
G
PR
LD XXX
g
SM
F
L
G
LD XXX
h
LD XXX
i

IX through XII), CDC2aLt remained concentrated within the apical dome, but its intensity declined significantly within the spikelet meristems (data not shown). In some cases within the terminal spikelet of the advanced double ridge apex, there were regions of higher expression along its edge towards its base (visible as circular zones) which preceded the differentiation of floret meristems. CDC2aLt expression within the lateral spikelet sites was concentrated on the adaxial (top) surface (see LD XXX apex in Figure 3.5g). Significant staining was observed in the floret meristems (see Figures 3.5f and 3.5g), which develop alternately along the spikelet axis.

Beginning at the advanced double ridge stage, expression of CDC2aLt was observed within the provascular region in the centre of the shoot apex (data not shown). Such vascular staining was also visible in the provascular traces leading to the glume (see LD XXX apex in Figure 3.5f) and lemma primordia (data not shown). Expression of CDC2aLt was also visible in young glume and lemma primordia, but fell to a low level (except in the provascular tissue) when they began to resemble leaf-like organs (see Figure 3.5f).

Staining for CDC2 mRNA and protein were comparable at LD XXX as shown in Figures 3.5g and 3.5h, respectively. The immunolocalisation negative control of antibody competed with synthetic peptide was weak as shown in Figure 3.5i, with cell wall staining seen in this photograph assumed to be non-specific. Expression of CDC2 protein therefore appears to be specific and is predominantly localised within the nucleus (see Figure 3.5h). At this stage (LD XXX), the terminal spikelet has yet to complete its differentiation, and CDC2 expression is concentrated in the epidermal and subepidermal layers (about 4 cells deep) as well as within the lemma primordia and the floret meristems.

In summary, both CDC2aLt message and CDC2 protein show similar staining patterns, increasing with the onset of flowering. Both transcript and protein are observed within the meristems (vegetative apical meristem, spikelet meristems and floret meristems) and initiating organ primordia. Whereas, stable CDC2 expression was observed within the meristems, expression in organs initiating from these primordia was more transient. Its expression in vegetative leaf primordia, however, was less transient than in the glume primordia (compare leaf primordia in Figures 3.5a (SD) or 3.5b (LD II) with glume primordia in Figure 3.5f). Beginning at LD XII, expression was detected within provascular tissue in the central core of the shoot apex as well as within glume and lemma primordia as they begin to elongate. Together these tissues represent the major regions of cell division of the shoot apical meristem.

Discussion

3.4 *Comparison of CDC2aLt with Homologues from Other Species*

3.4.1 *Nucleotide and Amino Acid Comparisons*

As a result of CDC2's indispensable role in cell division, this gene is highly conserved across all eukaryotes (John *et al.*, 1989). The putative full length translated *CDC2aLt* product is 33.9 kda in size and contains the perfectly conserved ATP binding region (GEGTYG, amino acids 11-16), the EGVPSTAIREISLLKE motif (amino acids 42-57), the HRDLKPQN (amino acids 125-132) and DFG (amino acids 146-148) domains typical of protein kinases and four tryptophan residues found to be conserved in all CDC2 kinases (see underlined motifs in Figure 3.2).

An alignment of the putative translated CDC2aLt protein with those from several plants (rice: CDC2Os-1, CDC2Os-2; maize: CDC2ZmA; snapdragon:

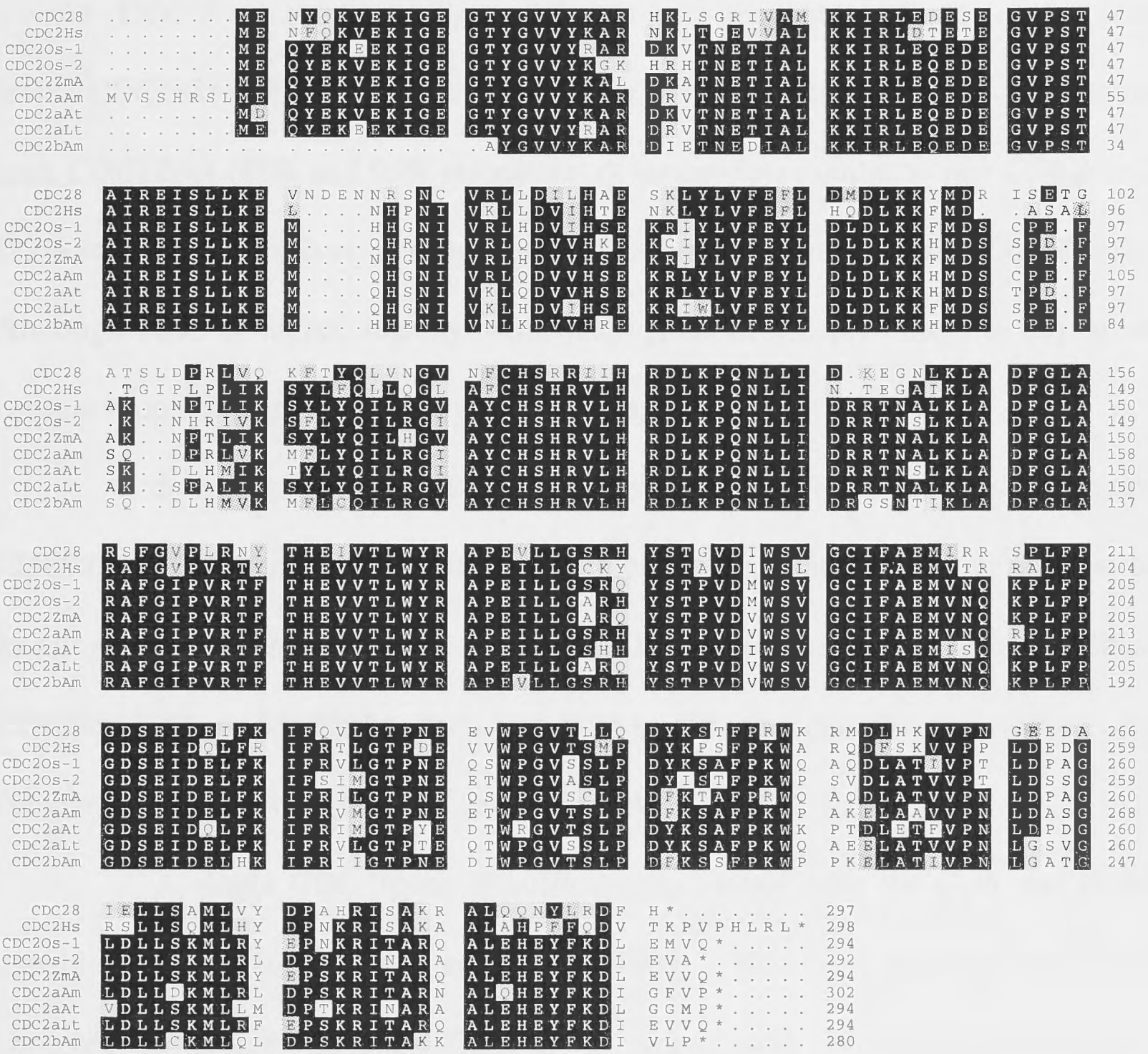


Figure 3.6 Comparison of the predicted amino acid sequence for CDC2aLt with CDC2 transcripts from other species.

Predicted amino acid sequences of CDC2 kinases from yeast (*Schizosaccharomyces pombe*; M12912), CDC28, human (P06493) CDC2Hs, rice (X60374) CDC2Os-1 and (X60375) CDC2Os-2, maize (M60526) CDC2ZmA, snapdragon (X97637) CDC2aAm and (X97638) CDC2bAm, *Arabidopsis thaliana* (X57839) CDC2a and *Lolium* CDC2aLt. Accession numbers are in brackets. Dots indicate gaps to maximise alignment. Amino acids that are identical in five or more of the proteins are blocked while those which are similar are shaded.

CDC2aAm, CDC2bAm; *Arabidopsis*: CDC2aAt) as well as a human (CDK1Hs) and a yeast (CDC28) is shown in Figure 3.6. At both the nucleotide and amino acid levels *CDC2aLt* is most similar to rice *CDC2Os-1* (90% and 97% respectively) and corn *CDC2ZmA* (88% and 95% respectively), though less similar to rice *CDC2Os-2* (78% and 92% respectively). (The Hind III site is not conserved in rice *CDC2Os-2*.) Although there is higher identity with other plant CDC2 proteins, the degree of conservation with the human and yeast proteins is also striking.

In a functional test for CDC2, both the rice CDC2Os-1 and the maize CDC2ZmA clones were shown to complement a temperature-sensitive mutant of budding yeast *cdc28* (Colasanti *et al.*, 1991; Hashimoto *et al.*, 1992). Since CDC2aLt is nearly identical to these, it would be expected that CDC2aLt would also complement this mutant, but this was not tested.

A single CDC2 gene is sufficient to control both the G_1/S and G_2/M transitions in yeast (Woollard and Nurse, 1995). However, multiple bands are observed on *Lolium* genomic DNA blots probed with various regions of the *CDC2aLt* gene (see Figure 3.3) and multiple CDK's have been cloned from maize, rice, *Antirrhinum* and *Arabidopsis* (Colasanti *et al.*, 1991; Hirayama *et al.*, 1991; Hashimoto *et al.*, 1992; Fobert *et al.*, 1994). Thus, the various CDK's may have differing and possibly overlapping roles within the cell cycle.

Differentially spliced CDC2 transcripts have not been reported previously. Since the position of the intron/exon boundaries for plant CDC2 genes have not been published previously, it is impossible to determine whether the 208 bp insertion found in two of the isolated *Lolium* CDC2 clones represents a conserved intron. The characteristics of its sequence (refer back to page 33), however, indicate it is likely an intron. Therefore the cDNAs containing this 208 bp insertion were

likely synthesised from incompletely processed transcripts. The mechanism whereby the clone with the 90 bp deletion was generated was not investigated. If this transcript is physiologically relevant, its translation product would lack the kinase domain (HRDLKPQN) as well as most of its C-terminus since the deletion causes a frame shift which would produce 6 new amino acids before premature termination of translation (see second translation line in Figure 3.2). It remains to be determined whether the truncated protein is translated.

3.4.2 *Comparison of CDC2aLt Expression With That for Other Plant CDC2a Genes.*

High expression of CDC2 genes (CDK's) cloned from maize, soybean, *Arabidopsis*, and *Antirrhinum* is found within the shoot apical meristem, whereas, low expression is found in mature organs (Colasanti *et al.*, 1991; Miao *et al.*, 1993; Fobert *et al.*, 1994, 1996; Segers *et al.*, 1996). Throughout vegetative and floral development, CDC2aLt was expressed in the shoot apical meristem, as assessed by *in situ* hybridisation (see Figures 3.5 a, b, d, e, and f).

At least two classes of CDK's have been cloned from several species including maize, rice, *Arabidopsis*, soybean and *Antirrhinum* (Colasanti *et al.*, 1991; Hirayama *et al.*, 1991; Hashimoto *et al.*, 1992; Miao *et al.*, 1993; Fobert *et al.*, 1994). In *Arabidopsis*, *Antirrhinum* and tobacco where the expression of CDK's has been analysed within specific phases of the cell cycle, it was determined that CDC2aAt, CDC2aAm, CDC2bAm and CDC2Nt1 were expressed at all phases, whereas CDC2bAt, CDC2cAm and CDC2dAm had a patchy expression and were only expressed in cells at particular phases of the cell cycle (Fobert *et al.*, 1994, 1996; Segers *et al.*, 1996; Setiady *et al.*, 1996). Since at any one time within the dome of the apex only ca. 10% of the cells are actively dividing (Ormrod and

Bernier, 1989), the uniform expression of CDC2aLt throughout the apical dome indicates that this gene is constitutively expressed throughout all phases of the cell cycle. CDC2aLt therefore marks cells within regions of the shoot apex with a high proliferative competence. Like CDC2aAt of *Arabidopsis* (Martinez *et al.*, 1992), CDC2aLt was expressed in provascular tissue (refer back to Figure 3.5f).

Activity of CDC2 protein at the G_2/M transition is highly regulated by phosphorylation at residues Thr14, Tyr15 and Thr161 or Thr167 (Lewin, 1990). These residues indicated in bold in Figure 3.2 are conserved in all CDC2 genes. Phosphorylation of Ser277 peaks during the G_1 phase and falls in S phase in vertebrate CDK's (Krek and Nigg, 1991). In *Lolium* as well as several other plants, Ser277 of CDC2a is conservatively changed to Thr277 (double underlined in Figure 3.2). Conservation of these key residues indicates that CDC2aLt may be postranslationally regulated by phosphorylation in a similar way to related vertebrate CDK's. Therefore, increased expression of CDC2 (mRNA or protein) does not imply an increased CDC2 activity or increased rate of cell division. This conclusion is supported by the fact that, the *Arabidopsis* homologue (*CDC2aAt*) is expressed within the shoot apical meristem of etiolated seedings, a condition in which the meristem does not actively divide (Hemerly *et al.*, 1993).

Although there is not an example of a mutant or transgenic plant which overproduces CDC2, a transgenic tobacco plant in which CDC2aAt message is cosuppressed does exist. Cosuppression of CDC2 message in this plant resulted in a reduced rate of cell division, however, although the plant was dwarfed in stature and was composed of fewer larger cells, its overall morphology was unchanged compared to an untransformed control (Hemerly *et al.*, 1995). This may indicate that additional controls (such as other CDK's with overlapping functions) help regulate

the overall rate of cell division in plants. Such additional controls might also be important in regulating cell division within the *Lolium* shoot apex during the vegetative to floral transition.

3.5 *CDC2aLt Expression and Flowering.*

Although CDC2aLt is not specifically expressed in dividing cells, it provides a marker for regions of the shoot apex with increased competence for cell division. Therefore, it was used as such following floral induction with a single LD. These results therefore extend and expand upon the patterns of mitotic activity at the shoot apex measured by Ormrod and Bernier (1990) and of Jacqmard *et al.* (1993) during the transition to flowering of *Lolium*.

Vegetative apices expressed CDC2aLt in the dome of the shoot apex and in the expanding leaf primordia at their base. Following the LD, apices of induced plants expressed CDC2aLt in more cell layers within the dome than uninduced plants (compare SD (a) and LD II (b) apices in Figure 3.5). High level expression of CDC2aLt within the apical dome was observed just after the LD and continued to be observed until glume stage was attained. The increased expression of CDC2aLt on the afternoon of LD II (see Figure 3.5b), occurred a few hours after translocation of the floral stimulus from the leaves to the apex would have been complete (Evans and Wardlaw, 1966; Evans, 1969) and at the same time at which other cellular changes such as increased uptake of ^{32}P into nucleic acids and ^{35}S into protein were observed (Rijven and Evans, 1967; Evans and Rijven, 1967). This increase in CDC2aLt expression was, however, after Jacqmard *et al.* (1993) detected a brief 5-fold increase in the mitotic index within the apical dome during the inductive LD (prior to movement of the floral stimulus out of the leaves and long before evocation was

achieved). Perhaps, post transcriptional modification of CDC2 activity or expression of another class of CDK is responsible for that transient increase in cell division.

Increases in CDC2aLt expression outside the dome of the apex were also observed. In the vegetative condition, little expression of CDC2aLt was observed in cells between the accumulated leaf primordia on the flanks of the shoot apex. However, by LD II (at the end of the LD) there was a continuous 3 cell layer deep band of CDC2aLt expression along the edge of the shoot apex (see LD II apex in Figure 3.5b). By LD IV (predouble ridge stage), the regions which will become future spikelet meristems exhibited high level expression of CDC2aLt, although the spikelet sites had not visibly expanded (see Figure 3.5d). Within the double ridge shoot apex at LD VI, CDC2aLt was no longer expressed along the entire edge of the shoot apex and was predominantly localised to circular zones of cells which would become the spikelet half of each double ridge (see LD VI apex in Figure 3.5e). This is the stage at which Knox and Evans (1968) recorded a 2 to 3-fold increase in RNA labelling as well as increased RNA staining within these sites (Knox and Evans, 1966). Reduced expression of CDC2aLt in the leaf primordia (see LD VI apex in Figure 3.5e) occurs concomitant with the sharp decrease in RNA staining seen at these sites by Knox and Evans (1966). Several histological changes have been recorded in both the dome and axillary buds of the shoot apex at this stage including an increase in the mitotic index (days 4 and 5; Ormrod and Bernier, 1990) as well as increased Feulgen staining (LD IV; Knox and Evans, 1966). Expression of CDC2aLt in both the dome and axillary buds of the shoot apex therefore anticipates a number of mitotic and metabolic changes which occur at the double ridge stage. Perhaps its expression creates the "natural synchrony" observed during the floral transition.

Two pieces of evidence argue against a general activation of the apex following LD floral induction. Firstly, the low expression of CDC2aLt within the central core of the shoot apex remains unchanged throughout development. The low CDC2aLt expression is supported by the low mitotic figures and weak incorporation of labelled substrates into RNA and protein within this region (Knox and Evans, 1968; Ormrod and Bernier, 1990). Secondly, although at the pre-double ridge stage CDC2aLt is continuously and transiently expressed on the edge of the shoot apex, at all other stages, CDC2aLt expression is localised to those regions which will divide rapidly within a few days. On the other hand, in *Lolium*, as is the case in wheat and in several other systems, during the transition to flowering there is an initial acceleration of the rate at which primordia are produced from the apical dome (Bernier, 1988; Evans and Blundell, 1996). In *Lolium*, this increased rate of primordia formation from the apical meristem begins on LD II and occurs for up to 6 days following the LD treatment (Evans and Blundell, 1996). This increased rate of primordium production is not specific to floral evocation since it also occurs in *Lolium* plants which are too young to flower in response to the inductive photoperiod (Evans and Blundell, 1996). Therefore, the increased rate of primordia production beginning on LD II (Evans and Blundell, 1996) is correlated with increased expression of CDC2aLt within the dome of the apex which might indicate that this increased expression of CDC2aLt is itself not evocation specific. Even so, expression, in tobacco, of a cDNA encoding for the yeast mitotic inducer *CDC25* under the 35S promoter caused precocious flowering and led to the initiation of more floral buds (Bell *et al.*, 1993).

From the present study, it is evident that *CDC2aLt* can be used as an effective marker of for spikelet site differentiation since its expression precedes their

visible differentiation, in much the same way that the expression of many of the homeotic genes (e.g., *AGAMOUS*) can be used as a marker for positions at which specific floral organs will develop. Furthermore, in *Arabidopsis* (a dicot), expression of CDC2aAt is independent of the phase of the cell cycle and is expressed at a much higher level in floral buds than in the rest of the plant (Martinez *et al.*, 1992). Thus, the increased rate at which primordia are produced from inflorescence (spikelets) and floral (floret) meristems compared with their vegetative counterparts correlates with the increased expression of these CDC2 species.

Possibly GA's are important in regulating these changes in CDC2 expression. Not only do applied GA's promote flowering of *Lolium* (Evans *et al.*, 1990), but the LD treatment probably leads to increased levels of active GA's. This result also fits with the finding of Evans and Blundell (1996) of an early increase in the rate of primordium initation following exposure to LD. Furthermore, in deepwater rice, applied GA increases CDC2Os-2 expression in association with an activation of the intercalary meristem (Sauter *et al.*, 1995). However, what is cause and what is consequence in such changes remains to be defined.

Conclusions

CDC2aLt, a single copy CDK gene from *Lolium* was isolated from a LD III florally induced PCR-based cDNA library. By *in situ* mRNA analysis, it was found to be expressed throughout the dome of the shoot apex as well as in the expanding leaf primordia at its base. Since only ca. 10% of cells within the apical dome are dividing at any time (Ormrod and Bernier, 1990), CDC2aLt expression must be a marker for competence for cell division. As early as LD II, the day after the florally inductive LD, expression of CDC2aLt increased within the apical dome. Although this is after a 5-fold transient increase in the mitotic index observed by Jacqmard *et al.* (1993), it correlates well with the time at which the rate of primordium production begins to increase (Evans and Blundell, 1996).

By LD III, although the spikelet primordia are not visible, CDC2aLt expression is observed in the axils of accumulated leaf primordia (the sites from which spikelets will initiate). This gene could therefore be used as a marker of spikelet initiation. CDC2aLt is also expressed in the floret meristems and provascular tissue. Both CDC2aLt protein and mRNA show a similar pattern of expression.

CHAPTER 4

EARLY EXPRESSED MADS-BOX GENES DURING FLORAL EVOCATION OF *LOLIUM*

Introduction

Studies of floral development in *Arabidopsis*, *Antirrhinum* and other species, led to the discovery that many mutants exhibiting homeotic transformations resulted from defects in MADS-box genes (Davies and Schwarz-Sommer, 1994). This gene family plays an important and well characterised role in floral development. Although many MADS-box genes are important in specifying floral meristem and floral organ identities, the expression of some members of this family is not exclusive to floral tissues (Rounsley *et al.*, 1995).

The MADS-box genes are a family of transcription factors with a conserved amino terminal domain of ca. 60 amino acids identified in its initial members which are transcription factors from yeast (***M****CM1*; which regulates mating-type determination and cell-type specific transcription), *Arabidopsis* (***A****GAMOUS* (*AG*); a C function gene involved in specifying stamen and carpel identities), *Antirrhinum* (***D****EFICIENS* (*DEF*); a B function gene involved in specifying petal and stamen identities), and humans (***S****RF*; a transcriptional regulator of c-fos) (Norman *et al.*, 1988; Passmore *et al.*, 1988; Sommer *et al.*, 1990; Yanofsky *et al.*, 1990). The first letter of each of these genes was used to coin the MADS acronym (Schwarz-Sommer *et al.*, 1990). This ancient family controls not only aspects of plant development, but also plays a regulatory role in eukaryotes as diverse as fungi and animals. It has even been suggested that a protein family which includes the *Escherichia coli* universal stress protein UspA represents an ancient progenitor of the MADS-box gene family (Mushegian and Koonin, 1996).

In addition to the highly conserved MADS-box domain, which is involved both in binding to DNA and dimerisation (Huang *et al.*, 1996; Pellegrini *et al.*, 1995), other regions of the protein are important in its functionality. C-terminal to the MADS-box domain lies a 33 amino acid linker (I) region (also known as the L region). Based on data from animal systems as well as recent data in *Arabidopsis*, the MADS-box domain in cooperation with the I region generates the DNA sequence specificity for each MADS-box factor (Olson *et al.*, 1995; Huang *et al.*, 1996; Krizek and Meyerowitz, 1996; Mizukami *et al.*, 1996; Reichmann *et al.*, 1996). This allows each MADS-box protein to activate a specific set of downstream target genes, either as homodimers (eg., AG or APETALA1 (AP1)) or as a heterodimers (eg., DEF/GLOBOSA (GLO)) with other coordinately expressed MADS-box genes (Schwarz-Sommer *et al.*, 1992; Huang *et al.*, 1993; Davies *et al.*, 1996; Reichmann *et al.*, 1996). C-terminal to the I region lies the K-box, exhibiting structural homology with the coiled-coil domain of keratin. This domain is believed to form two or three amphipathic α-helices thought to be involved in protein-protein interactions (Ma *et al.*, 1991). Dimerisation between MADS-box proteins is mediated by this domain (Davies *et al.*, 1996). The C-termini of this gene family are less related, but tend to be rich in acidic amino acids which are characteristic of transactivation domains. Neither the K-box nor the C-terminal domain are required for DNA binding (Huang *et al.*, 1996). In summary, the N-terminal end of the protein (MADS-box and I region) is involved in DNA binding, with the K-box mediating dimerisation and the C-terminus involved in transactivation.

Several lines of evidence indicate that MADS-box genes are transcription factors. These proteins have one or several nuclear localisation signals

(NLS's), regions of basic amino acids (McGonigle *et al.*, 1996). The absence of extranuclear localisation signals also supports their localisation within the nucleus. Additionally, several MADS-box proteins bind to the CArG-box ($CC(A/T)_6GG$) DNA sequence motif, present in promoters of their downstream target genes (Sommer *et al.*, 1990; Schwarz-Sommer *et al.*, 1992; Huang *et al.*, 1993; Shiraishi *et al.*, 1993; Huang *et al.*, 1995; Reichmann *et al.*, 1996). Only MADS-box proteins which can dimerise and are successfully imported into the nucleus are available to bind such DNA recognition sequences (McGonigle *et al.*, 1996). Since the sequence specificity (Huang *et al.*, 1993; Huang *et al.*, 1995) and affinity for a specific sequence (Reichmann *et al.*, 1996) differs for each interacting pair of proteins, so too does the set of target genes regulated by that pair. The fact that many MADS-box proteins seem to interact at the protein level, but that only some combinations are imported into the nucleus or bind DNA with measurable sequence specificity, might represent a form of post-transcriptional regulation. Although ternary interactions between specific MADS-box dimers and coordinately expressed proteins outside this family (non-MADS-box genes including KNOTTED-1, BELL1, APETALA2, LEUNIG and LEAFY) have not been reported in plants, such interactions may also help define the specific set of targets which are activated and may even allow CArG-box-non-binding pairs to become active transcriptional regulators.

The number of MADS-box genes varies enormously between species. In *Arabidopsis* best estimates indicate that there are about 30 members, with potentially double that number present in maize (Mena *et al.*, 1995; Theißen and Saedler, 1995), an ancient tetraploid (Goodman *et al.*, 1980). All members display reasonable homology within the N-terminal region (MADS-box, I region and K-box), however,

even between true homologues, the C-terminal domain can be enormously divergent (Rounsley *et al.*, 1995). The remainder of this chapter will focus on two members of the SQUA branch of the MADS-box family, *AP1* and *AGL8*, which in *Arabidopsis* are expressed early in the vegetative to floral transition.

The first member of the SQUAMOSA (SQUA) branch of the MADS-box family was isolated by transposon tagging of the *squa* floral homeotic mutant of *Antirrhinum* (Huijser *et al.*, 1992). The *SQUA* homologue from *Arabidopsis* was identified as ***AGAMOUS Like 7*** (*AGL7*), a MADS-box containing gene which by RFLP analysis mapped to the *AP1* locus (Mandel *et al.*, 1992). Both mutants are characterised by vegetative features in developmental regions where flowers would normally be found. For instance, the *squa* mutant is characterised by excessive bract formation with shoots forming where flowers would normally form, resulting in few and often incomplete flowers (Huijser *et al.*, 1992). Mutations in the homologous gene, *ap1*, also shows inflorescence characteristics in the flowers including leaf-like first whorl organs often subtended by secondary, and even higher order, flowers in their axils (Mandel *et al.*, 1992).

Ectopic overexpression of AP1 under the control of the viral 35S promoter (35Sp), produces a *terminal flower* phenocopy (Mandel and Yanofsky, 1995a). This indicates that in *Arabidopsis*, AP1 is sufficient to initiate floral development. However, additional genes are necessary for normal floral development since, (i) flowering in these overexpressors does not occur immediately upon germination, (ii) flowers of the overexpressors, although largely normal, display abnormalities characteristic of *lfy* mutant plants and (iii) *ap1* mutants are predominantly floral in their characteristics (Huijser *et al.*, 1992; Mandel *et al.*, 1992; Mandel and Yanofsky, 1995a).

In addition to the function of *AP1* and *SQUA* in floral meristem identity, mutant and expression data indicate that these genes are also involved in specifying the identity of sepals and petals (Bowman *et al.*, 1993). Expression of ZAP1, in the sterile organs of the ear and tassel suggests that this role is conserved in maize (Mena *et al.*, 1995). This study will examine the expression of two *Lolium* AP1-like genes by *in situ* hybridisation.

In the first report of MADS-box genes being involved in the vegetative to floral transition in an inducible system, using the LDP *Sinapis alba*, three MADS-box genes (*SaMADS A*, *B* and *C*) were shown to be expressed sequentially (Menzel *et al.*, 1996). Expression of these genes was not detectable prior to the LD floral induction. Expression of homologues of these genes in *Sinapis* has been analysed in other systems. For instance, *SaMADS A* is homologous with *TobMADS1*, a tobacco gene isolated from a day neutral variety and is expressed in both vegetative and floral tissue (Mandel *et al.*, 1994). *SaMADS A* is also homologous with *TM3*, a tomato gene which is also expressed in leaves and transition meristems (eg., meristems of the *anantha* mutant) (Pnueli *et al.*, 1991). *SaMADS B* of *Sinapis* is homologous to *AGL8* of *Arabidopsis* (Mandel and Yanofsky, 1995b). This *Arabidopsis* gene was isolated in a low stringency screen of a cosmid library (genomic) probed with a vegetatively expressed member of the MADS-box family (*AGL3*). Its corresponding cDNA was isolated from an above-ground-tissue cDNA library (Mandel and Yanofsky, 1995b). Expression of AGL8 mRNA is first observed in the inflorescence apex when the plant begins to bolt (Mandel and Yanofsky, 1995b).

It is clear, particularly from the studies of mutants, that MADS-box genes are important regulatory factors both during the vegetative to floral transition as well as in specifying floral meristem and floral organ identities. Therefore in the

studies reported here, a screen was initiated to identify MADS-box genes expressed at the shoot apex during the vegetative to floral transition in *Lolium*. Two questions were posed. Firstly, are MADS-box genes involved in the vegetative to floral transition in the LD-inducible plant *Lolium temulentum* strain Ceres, as they are in the LD-inducible plant, *Sinapis alba*? Secondly, if they are, what is their timecourse of expression in the shoot apex during this transition?

Results

4.1 *Cloning of Two AP1-Like Genes from Lolium*

Initially, PCR was used to clone a fragment of the MADS-box domain from *Lolium* genomic DNA. Amplification of a 129 bp (43 amino acid) fragment from *Lolium* genomic DNA used a touchdown PCR program. and employed a degenerate oligonucleotide primer pair. MADS-1 [5'-d(CG**GAATTC**ATGGGNM-GNGGNAARRT)-3'], was identical to nucleotides 1 through 17 (underlined), with MADS-43 [5'd(CG**GGATCC**IACYTGIGCRTCRCAIARIAC)-3'] being complementary to nucleotides 111 through 129 (underlined; designed by Dr Steve Strauss, Oregon State University, Corvallis, OR, USA). This fragment was gel purified, its ends digested with EcoR I and BamH I (restriction endonuclease sites indicated in bold above) and cloned into pBluescript SK(+) plasmid vector which had been digested with the same restriction endonucleases. Four 129 bp MADS-box fragments were sequenced and found to represent two distinct species, termed LtMADSf1 and LtMADSf2 (see Figure 4.1 a). Both LtMADSf1 and LtMADSf2 were most similar at the amino acid level to the homologous region in AP1/SQUA homologues from several species including corn, *Sinapis*, *Antirrhinum*, and

Arabidopsis (Blast scores >200; see Figure 4.1 b and c) with these regions being less related to other MADS-box containing genes (Blast scores <170; not shown).

The degenerate PCR-generated MADS-box fragments (see Figure 4.1a) were used to probe a virtual northern blot of cDNA from vegetative (SD) and florally evoked (LD III) apices at intermediate stringency (0.5 X SSPE; 0.2% SDS at 65°C). At least two bands representing MADS-box containing transcripts were observed in cDNA from both vegetative and florally evoked apices, with the intensity of the hybridising bands found to increase following floral induction (see Figure 4.2).

Since the expression of at least two MADS-box containing transcripts increased soon after LD floral induction, the same probe at the same stringency was used to screen duplicate lifts of a PCR-based cDNA library constructed from *Lolium* LD III apex material. (See section 2.2 for the detailed method for PCR-based library construction and screening.) Approximately 30 plaques per 50 000 were found to hybridise with the degenerate MADS-box probe. These fell into three size classes of 0.8, 1.1 and 1.2 kbp. To identify and correct for PCR-based errors within the library cDNAs, three inserts of each size class were sequenced and the consensus sequence determined. Within each size class, each clone originated from a different cDNA, since they varied in the length of their 5'-UTR's (which ranged from 85 to 170 nucleotides) and the length of their poly A tails (which ranged from 4 to 18 nucleotides). Two genes termed *Lolium temulentum MADS1* (*LtMADS1*) and *LtMADS2* were represented by the three cDNA size classes (see Figures 4.3 and 4.4 respectively). LtMADS1, 1.2 kbp, is 79% identical at the nucleotide level to LtMADS2, 1.1 kbp, with these genes being 88% similar and 78% identical at the amino acid level. The LtMADSf1 and LtMADSf2 PCR products were identical to

Figure 4.1a. Nucleotide and translated protein sequence for two MADS-box fragments amplified with the MADS1/MADS43 degenerate primer pair.

LoMADSf1

```
1   ATGGGGCGCGGCAAGGTGCAGCTCAAGCGGATCGAGAACAAGATCAAC
1    M  G  R  G  K  V  Q  L  K  R  I  E  N  K  I  N
49  CGCCAGGTCACCTTCTCCAAGCGCCGCTCAGGCCTGCTCAAGAAGGCG
17   R  Q  V  T  F  S  K  R  R  S  G  L  L  K  K  A
97  CACGAGATCTCCGTGCTCTGCGACGCAGAGGTC
33   H  E  I  S  V  L  C  D  A  E  V
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LtMADSf2

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1   ATGGGTCGCGGCAAGGTGCAGCTGAAGCGGATAGAGAACAAGATAAAC
1    M  G  R  G  K  V  Q  L  K  R  I  E  N  K  I  N
49  CGTCAGGTGACATTCTCCAAGCGCCGCAACGGGCTACTCAAGAAGGCG
17   R  Q  V  T  F  S  K  R  R  N  G  L  L  K  K  A
97  CACGAGATCTCCGTCCTCTGCGACGCCGAGGTC
33   H  E  I  S  V  L  C  D  A  E  V
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b. Translated protein sequence of LtMADSf1 and 2 compared to this region of *Arabidopsis AP1*.

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LtMADSf1    1 MGRGKVQLKRIENKINRQVTFSKRRSGLLKKAHEISVLCDAEV 43
LtMADSf2    1 .........................N.................. 43
AP1         1 ....R.....................S.................. 43
```

c. Blast search of LoMADSf1 against the PIR genebank.

Sequences producing High-scoring Segment Pairs:		High Score	Smallest Sum Probability P(N)	N
TM4	tomato AP1 homologue	210	2.6e-23	1
POTM1	potato AP1 homologue	210	3.8e-23	1
SQUA	snapdragon AP1 homologue	207	1.0e-22	1
SaMADS C	mustard AP1 homologue	207	1.1e-22	1
AP1	Arabidopsis AP1	207	1.1e-22	1

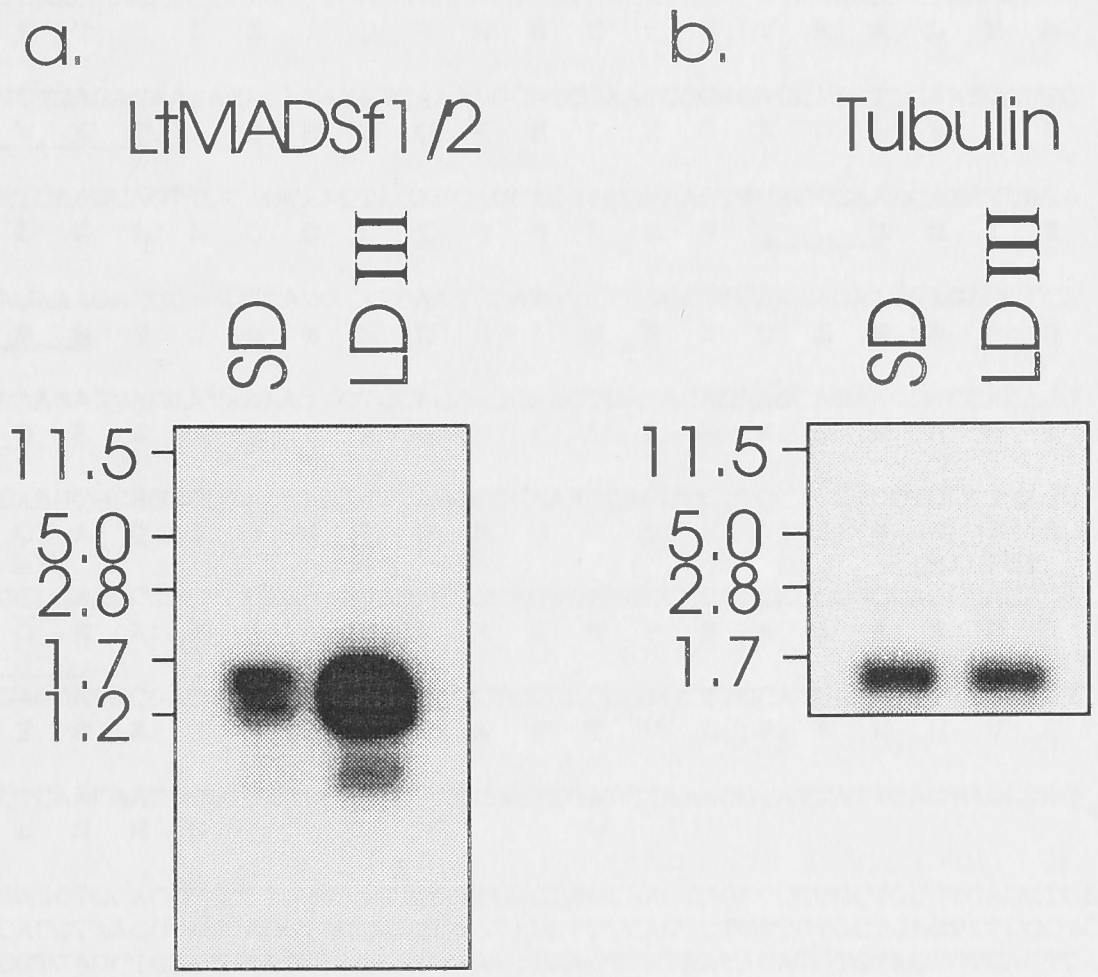

Figure 4.2 Virtual northern blot analysis of vegetative SD and florally induced LD III PCR-based cDNA hybridised with (a) a degenerate LtMADSf1 and LtMADSf2 MADS-box probe and (b) a fragment of a *Lolium* tubulin cDNA (see Table 2.3.4) to compare loading between lanes. Sizes in kbp are indicated to the left.

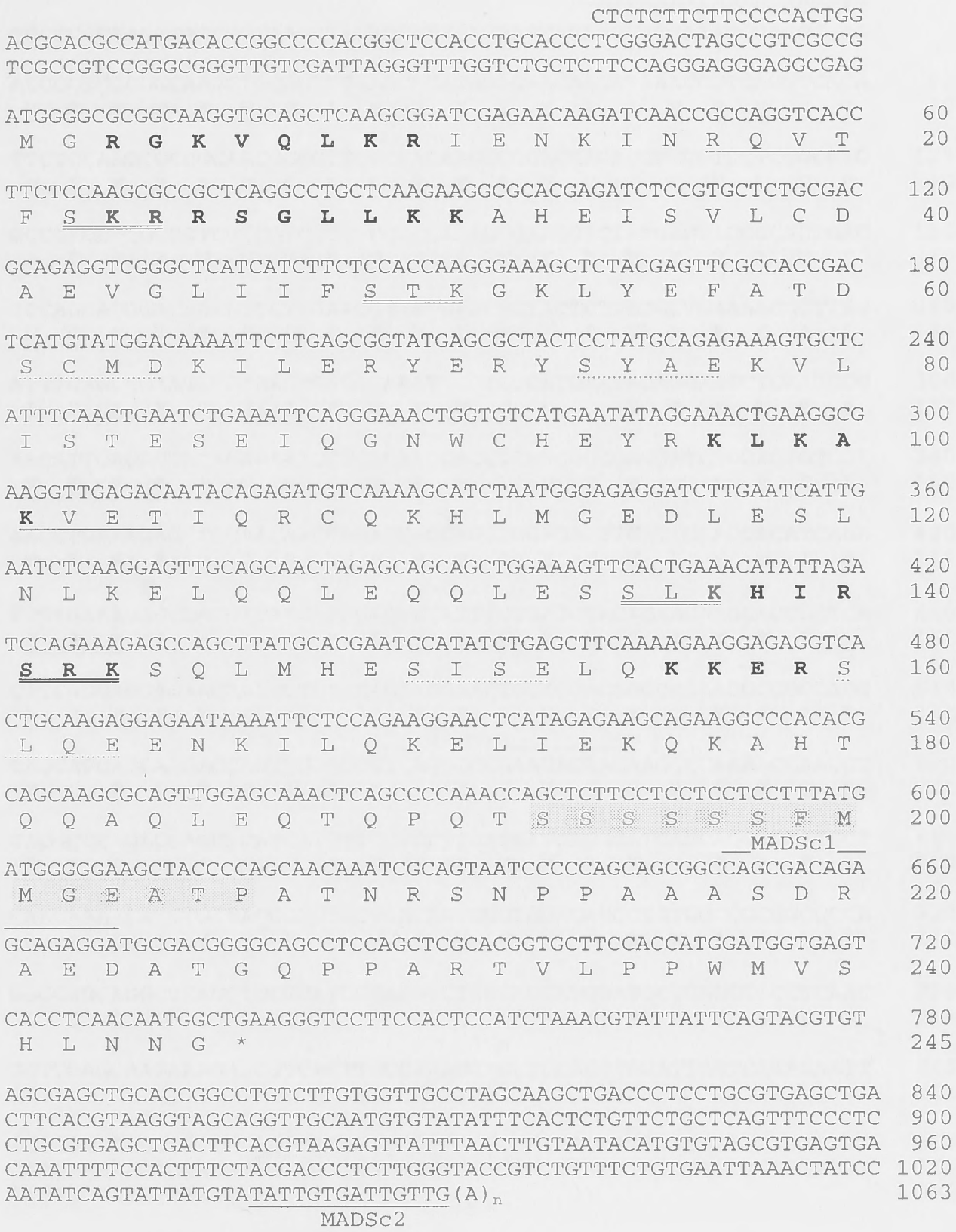

Figure 4.3 Nucleotide and predicted amino acid sequence of *LtMADS1*.

Numerals to the right of each sequence indicate nucleotide and amino acid positions with the initiation codon providing the start. Gene-specific primers for a fragment at the 3'-end MADSc1 and MADSc2 are indicated above the nucleotide sequence. Putative nuclear localisation signals are shown in bold. Putative sites for calcium-dependent phosphorylation are underlined, with those for phosphorylation by casein kinase double underlined and those for CK2 phosphorylation with a dotted underline. The hatched sequence is a putative STP transcriptional activation domain.

Figure 4.4 Nucleotide and predicted amino acid sequence of *LtMADS2*.

Numerals to the right of each sequence indicate nucleotide and amino acid positions with the initiation codon providing the start. Gene-specific primers for a fragment at the 3'-end MADSr1 and MADSr2 are indicated above the nucleotide sequence. Putative nuclear localisation signals are shown in bold. Putative sites for calcium-dependent phosphorylation are underlined, with those for phosphorylation by casein kinase double underlined and those for CK2 phosphorylation with a dotted underline. The arrowhead at nucleotide 430 indicates the site of a 20 nucleotide insertion which is retained in the alternatively-spliced LtMADS2' transcript.

```
CTTGGTAGACAGCCGGAAGCTAGATCAGGGAGAATCAGAGGCACGAAATCGAGGCAAG

ATGGGTCGCGGCAAGGTGCAGCTGAAGCGGATAGAGAACAAGATAAACCGTCAGGTGACA     60
 M  G  R  G  K  V  Q  L  K  R  I  E  N  K  I  N  R  Q  V  T      20

TTCTCCAAGCGCCGCAACGGGCTACTCAAGAAGGCGCACGAGATCTCCGTCCTCTGCGAC    120
 F  S  K  R  R  N  G  L  L  K  K  A  H  E  I  S  V  L  C  D      40

GCCGAGGTCGCCGTCGTCGTCTTCTCCCCGAAAGGGAAGCTCTATGAGTACGCCACTGAC    180
 A  E  V  A  V  V  V  F  S  P  K  G  K  L  Y  E  Y  A  T  D      60

TCCAGCATGGACAAAATTCTTGAACGTTATGAACGCTACTCTTATGCTGAAAAGGCTTTG    240
 S  S  M  D  K  I  L  E  R  Y  E  R  Y  S  Y  A  E  K  A  L      80

ATTTCAGCTGAATCTGAAAGTGAGGGAAATTGGTGCCATGAATACAGGAAGCTGAAGGCG    300
 I  S  A  E  S  E  S  E  G  N  W  C  H  E  Y  R  K  L  K  A     100

AAGATTGAGACTATACAAAAATGTCGCAAGCACCTCATGGGGGAGGATCTGGAGTGTCTA    360
 K  I  E  T  I  Q  K  C  R  K  H  L  M  G  E  D  L  E  C  L     120

AACCTGAAAGAGCTCCAACAACTAGAGCAGCAGCTGGAGAGTTCATTGAAGCACATCAGA    420
 N  L  K  E  L  Q  Q  L  E  Q  Q  L  E  S  S  L  K  H  I  R     140
          |←      20 bp      →|
TCGAGAAAGGTTGTTATTTATCAACTTAGAGCCACCTTATGATGGAGTCCATTTCTGAGC    480
 S  R  K  V  V  I  Y  Q  L  R  A  T  L  *                       153

TACAGAAGAAGGAGCGGTCACTCCAGGAGGAGAACAAGGCTCTACAGAAGGAACTGGTGG    540
AGAGGCAGAAGGCGGCCAGGCAGCAGCAGCAAGAGCAGTGGGACCGTCAGACCCAAACAC    600
AACAAGCCCAAAACCAACCTCAGGCCCAGACGAGCTCATCATCTTCCTCCTTCATGATGA    660
GGGATCAGCAGGCCCATGCTCAACAAAACATCTGTTACCCGCTGGTGACAATGGGTGGAG    720
AGGCTGTGGCCGCGGCGCCAGGGCAGCAGGGGCAGCTTCGCAT      763
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Figure 4.5 Nucleotide and predicted amino acid sequence of LtMADS2'.

Numerals to the right of each sequence indicate nucleotide and amino acid positions with the initiation codon providing the start. The 20 nucleotide insertion which is absent in LtMADS2 transcripts is indicated between the lines (nucleotide 430 to 449).

the coding region for amino acids 1 to 43 from LtMADS1 and LtMADS2 respectively (compare Figure 4.1a with Figures 4.3 and 4.4).

The third species is a truncated and differential spliced version of the LtMADS2 transcript and was termed LtMADS2' (see Figure 4.5). The LtMADS2' sequence, 0.8 kbp, was identical to LtMADS2 throughout the MADS-box, I and K-box regions. A 20 bp insertion is present after the sequence coding for the K-box (see Figure 4.5). As determined from genomic sequencing in this region, the insertion results from differential processing of intron 4, due to a 268 nucleotide intron which has two 5' donor sites, separated by 20 nucleotides, and a single 3' acceptor site, (see Figure 4.6). The LtMADS2 transcript is produced when the full 268 nucleotide intron is removed, producing a putative translated product of 261 aas with a Mr of 30.0 Kda and a pI of 9.51. The LtMADS2' transcript results from incomplete removal of the full 268 nucleotide intron, a 20 nucleotide intron fragment being retained between the 5' donor sites. This retained intron fragment changes the reading frame, resulting in the addition of 10 new amino acids before translation terminates prematurely at the end of the K-box. The predicted size of this second product is 160 amino acids with an Mr of 17.9 Kda and a pI of 10.12. It remains to be determined whether both the full length and truncated messages are translated.

4.2 *Genomic DNA Gel Blot Analysis of LtMADS1 and LtMADS2*

Gene fragments specific for the 3'end including the UTR for each of *LtMADS1* and *LtMADS2*, were generated by PCR. The *LtMADS1* specific fragment (353 bp) was generated with the primers MADSc1 [5'-d(CAGCGACAGA-GCAGAGGA)-3'] and MADSc2 [5'-d(TTTTCAACAATCACAATA)-3'], whilst the *LtMADS2* specific fragment (307 bp) was generated with the primers MADSr1 [5'-d(CAGACCCAAACACAACAA)-3'] and MADSr2 [5'-d(GGCAAGCAAACAT

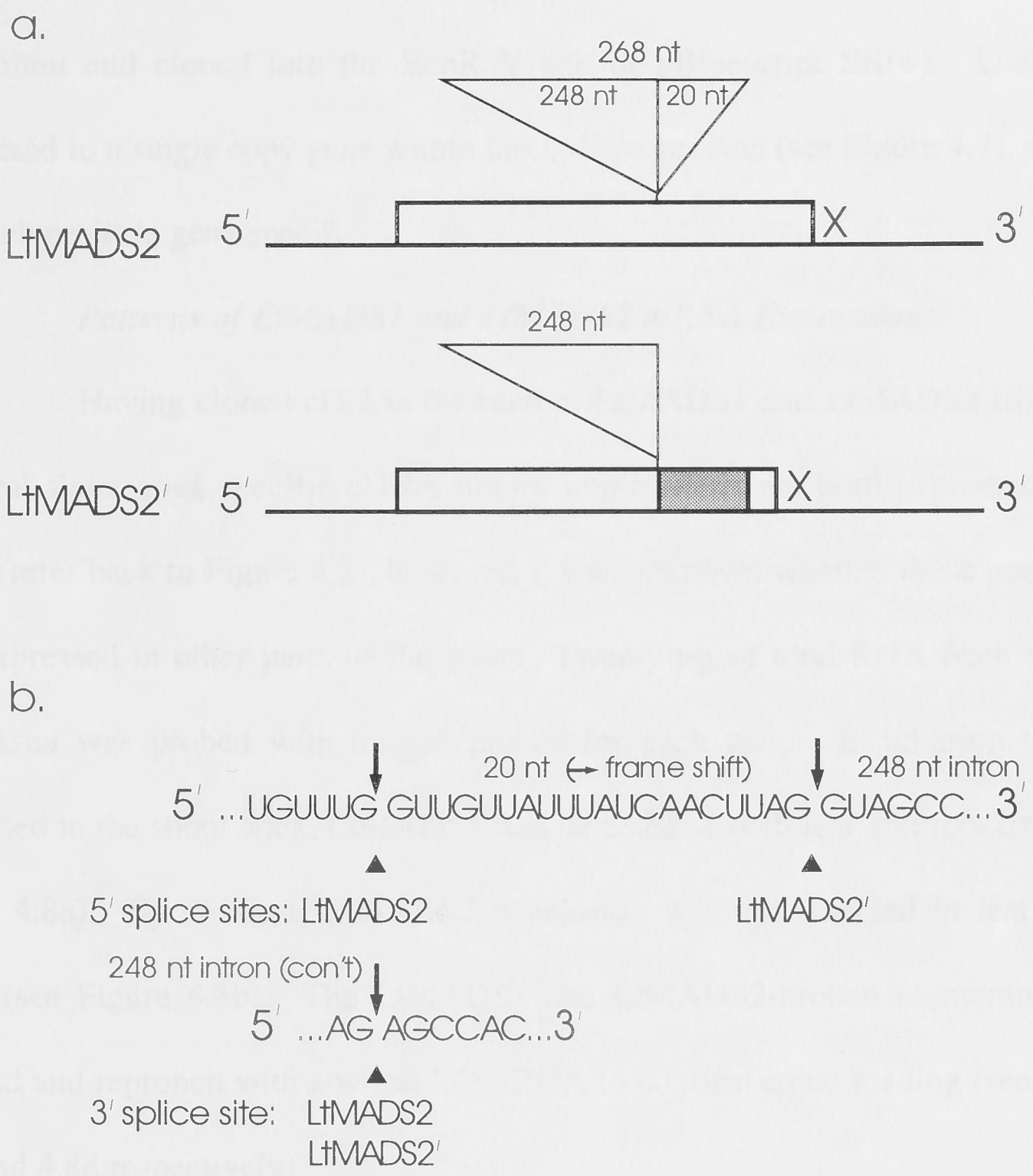


Figure 4.6 Scheme of alternative splicing events used to generate LtMADS2 and LtMADS2' transcripts.

(a) The boxed sequence indicates the open reading frame (ORF) with stop codons indicated with an X and untranslated regions (UTR) as lines. Changes in the amino acid sequence of the putative translated protein from LtMADS2' transcripts compared to LtMADS2 transcripts is indicated by the filled box. Spliced introns are shown as open triangles. (b) Sequence of the putative pre-mRNA in the intron 4 region determined by genomic sequencing. Putative splice sites for LtMADS2 and LtMADS2' transcripts are indicated with arrows. nt is nucleotides.

-AACTA)-3'] (primer locations indicated on Figures 4.3 and 4.4). These fragments were blunt end cloned into the EcoR V site of pBluescript SK(+). Each probe hybridised to a single copy gene within the *Lolium* genome (see Figure 4.7), showing that each probe is gene specific.

4.3 *Patterns of LtMADS1 and LtMADS2 mRNA Expression*

Having cloned cDNAs for each of LtMADS1 and LtMADS2 from a LD III floral shoot apex specific cDNA library implies they are both expressed in this tissue (refer back to Figure 4.2), however, it was unknown whether these genes were also expressed in other parts of the plant. Twenty µg of total RNA from root and leaf tissue was probed with unique probes for each gene. In addition to being expressed in the shoot apex, LtMADS1 was detected in both leaf and root tissue (see Figure 4.8a). By contrast, LtMADS2 expression was not detected in leaf or root tissue (see Figure 4.8b). The LtMADS1 and LtMADS2-probed membranes were stripped and reprobed with a wheat 26S rRNA to confirm equal loading (see Figures 4.8c and 4.8d respectively).

To determine the temporal (vegetative through LD XXX) and spatial localisation of these mRNAs within the apex following floral induction, *in situ* hybridisation was performed on tissue sections as detailed in Appendix I. Expression of LtMADS1 and LtMADS2 were analysed using DIG-labelled riboprobes synthesised for each 3' gene specific sequence (see section 4.2). Due to the fact that T7 ribopolymerase is more efficient than either the SP6 or T3 ribopolymerases, sense and antisense riboprobes for each gene were generated from the T7 promoter. To enable template production for synthesis of the sense riboprobes, the PCR-generated 3' gene specific fragments were recloned as EcoR I/Hind III fragments (which flank the EcoR V site) from pBluescript SK(+) into

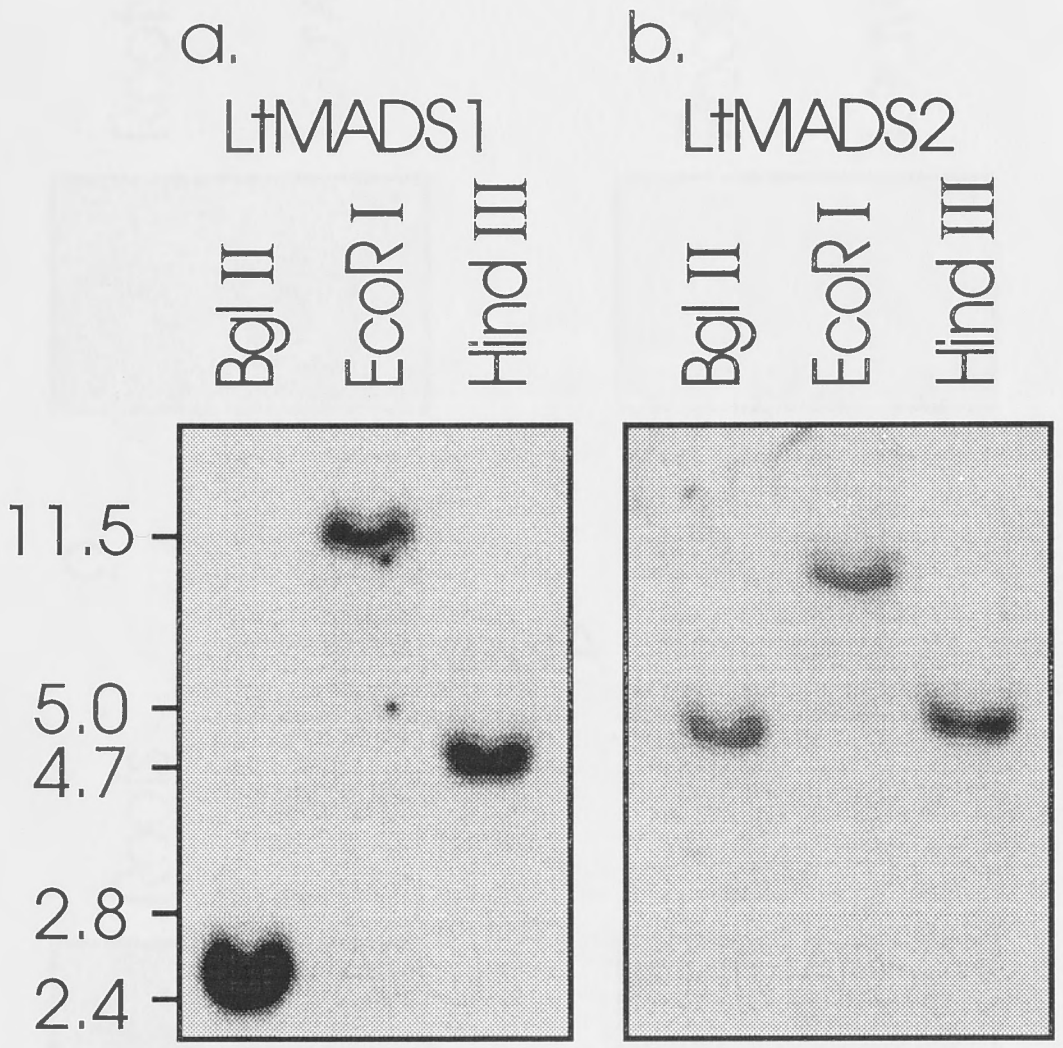


Figure 4.7 *Lolium* genomic DNA gel blot analysis with gene-specific probes for *LtMADS1* and *LtMADS2*.

Genomic DNA (20 µg) was digested with Bgl II, EcoR I and Hind III. After blotting, the digested DNA was hybridised with (a) a gene specific probe for *LtMADS1* (MADSc1/2; nucleotide 650 to 1062) or (b) a gene specific probe for *LtMADS2* (MADSr1/2; nucleotide 568 to 874). Sizes in kbp are indicated to the left.

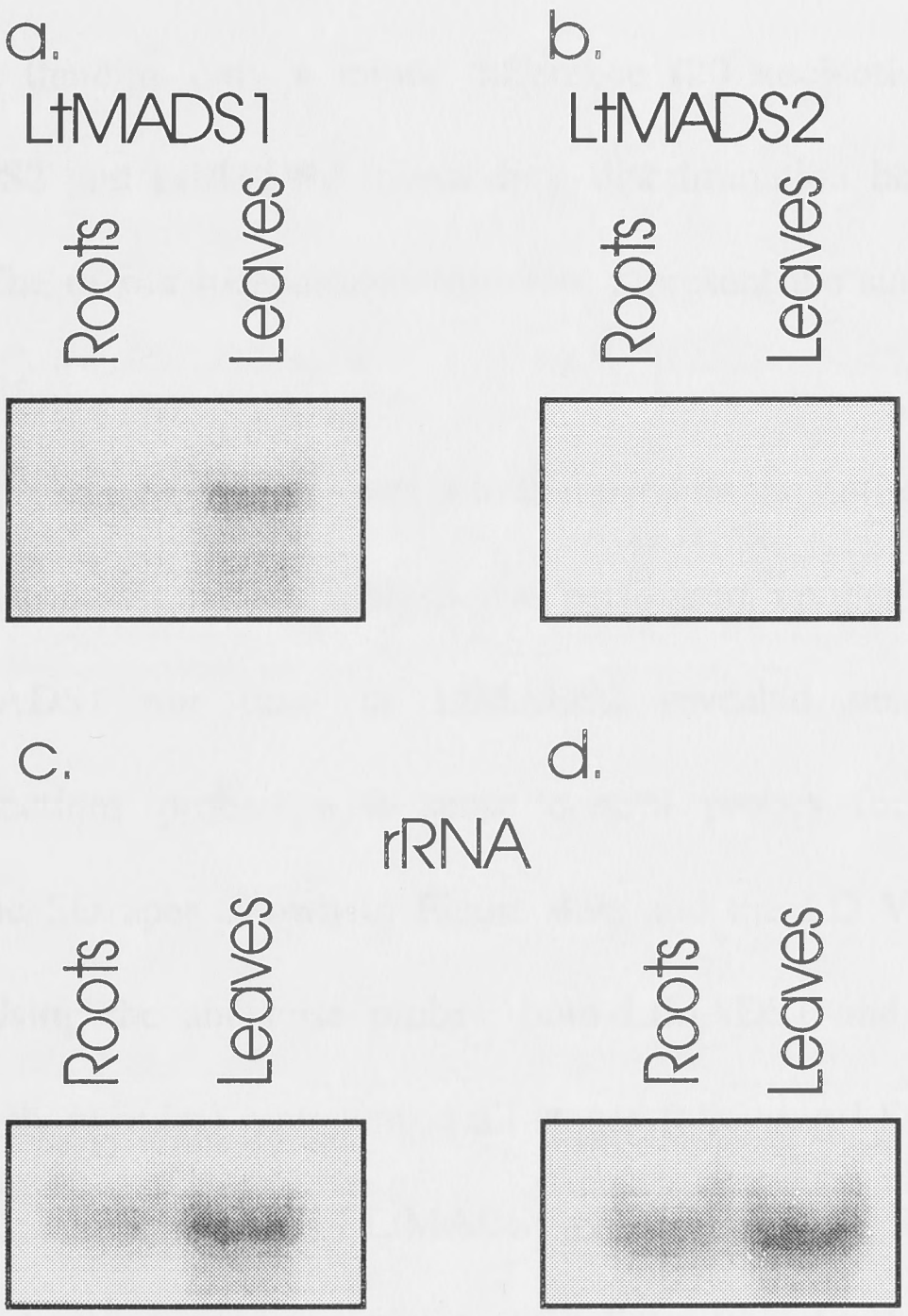


Figure 4.8 RNA gel blot analysis of LtMADS1 and LtMADS2 expression in leaves and roots.

After blotting, total RNA (20 µg) from leaves or roots was hybridised with (a) a gene specific probe for *LtMADS1* (MADSc1/2; nucleotide 650 to 1062) or (b) a gene specific probe for *LtMADS2* (MADSr1/2; nucleotide 568 to 874). Blots (a) and (b) were stripped and reprobed (c) and (d) respectively with a 9-kb wheat 26S rRNA clone, pTA71 (Gerlach and Bedbrook, 1979).

pBluescript KS(+), in which the multiple cloning site is reversed. Colorimetric detection of the hybridising probes was via an anti-DIG Fab fragment conjugated to alkaline phosphatase.

Since there is only a minor difference (20 nucleotides) in sequence between LtMADS2 and LtMADS2' transcripts, discrimination between these was not attempted. The *in situ* localisations therefore represent the summed expression for both transcripts.

At a probe concentration equal to that used on the antisense slides and at all times for which *in situ* mRNA analysis was performed, neither the sense control probe for LtMADS1 nor that for LtMADS2 revealed detectable staining. Representative sections probed with sense control probes for LtMADS1 and LtMADS2 are the SD apex shown in Figure 4.9a and the LD VI apex shown in Figure 4.10c. Using the antisense probes, both LtMADS1 and LtMADS2 were expressed in the shoot apical meristem at all stages following LD floral induction. At all stages analysed, expression of LtMADS1 greatly exceeded that of LtMADS2. This was evident in the increased time required for colour development using the LtMADS2 probe (2 days) compared with using the LtMADS1 probe (8 h).

In the vegetative (SD) shoot apex, LtMADS1 was predominantly expressed in the epidermal and subepidermal layers (ca. 3 or 4 cell layers deep) as well as in the tips of the developing leaf primordia (see SD apex in Figure 4.9b). The tip of this shoot apex is in the median plane, however, its base is not. In this section, every bump on the flanks of the shoot apex is a leaf primordium and not a spikelet site. At the vegetative stage, LtMADS2 was expressed at a low level in apical dome of the shoot apex (to a depth of 3 cell layers), but was largely excluded from the leaf primordia (see SD apex in Figure 4.10a). The presence of LtMADS1

and absence of LtMADS2 expression in the accumulated leaf primordia determined by *in situ* hybridisation, supports the presence of LtMADS1 and absence of LtMADS2 in leaf tissue (refer back to Figures 4.8a and 4.8b respectively). By LD III, the expression of LtMADS2 becomes expressed in more cell layers within the apical dome and subsequently in the epidermal and subepidermal layers along the length of the shoot apex. Until LD IV, when the spikelet sites began to expand in the axils of existing leaf primordia, the expression pattern of LtMADS1 was similar to that in the SD vegetative shoot apex (data not shown).

Upon attaining the double ridge stage, LtMADS1 expression remained concentrated around the edge of the shoot apical meristem (epidermal and subepidermal layers), however, three distinct regions within the shoot apex became visibly defined by the level of LtMADS1 expression (see LD IV apex of Figure 4.9c). Expression was greatest within the dome of the shoot apex as well as within the developing spikelet primordia, seen as circular zones of expression within the shoot apical meristem (indicated with arrowheads). Less expression of LtMADS1 was observed in the leaf primordia subtending the spikelet sites (see Figure 4.9c). Lowest expression was observed in the central core of the shoot apical meristem. At a somewhat later stage, LtMADS2 expression was detected only in the shoot apical meristem and within the spikelet primordia, with little detectable expression in the regressing leaf primordia (see LD VI apex of Figure 4.10d).

At the advanced double ridge stage occurring between LD IX (data not shown) and LD XII significant changes in the expression of both LtMADS1 and LtMADS2 were evident. At this time, each spikelet site (including the terminal dome) was assuming an elongated morphology reminiscent of the structure of the tip

Figure 4.9 *In* situ localisation of LtMADS1 transcripts in *Lolium* shoot apices.

In situ hybridisations were performed on longitudinal sections of wax embedded apices at different developmental stages using DIG-labelled *in vitro*-transcribed riboprobes synthesised from a gene specific fragment of the LtMADS1 cDNA (MADSc1/2; nucleotide 650 to 1062). Sectioned apices (a) to (f) were from experiment Lt417 with sections (g) and (h) from experiment Lt434 (see Figure 2.1). Sections (b), (c), (d), (e), (f), (g) and (h) were hybridised with the antisense riboprobe. A representative section hybridised with the sense control riboprobe is shown in (a). Hybridisations were with an equivalent sense or antisense probe concentration. The same dilution of anti-DIG antibody was used to detect riboprobe hybridisation and the duration of the colour development was identical for all sections in this figure. Hybridisation is visible as the pink to purple product of the alkaline phosphatase reaction. Photographs were taken using Nomarski optics with identical exposures. Therefore, in all antisense hybridised sections, the intensity of staining is directly proportional to the expression of LtMADS1. Median sections through (a,b) six-week old vegetative (SD) shoot apices (c) LD IV pre-double ridge shoot apex, (d) lateral spikelet sites on the flanks of the shoot apex at glume stage (LD XII), (e) lateral spikelet sites on the flanks of the shoot apex at floret stage (LD XXI), (f) terminal spikelet site subtended by two glume primordia with three floret meristems alternately along the terminal spikelet axis subtended by lemma primordia (LD XXX), (g) three lateral spikelet sites at floret stage (LD XXX) and (h) a lemma, stamen and carpel primordium (left to right) on the flank of a floret site (LD XXX). The bars are 50 μm.

Abbreviations: AM, apical meristem; AS, anterior stamen primordium; C, carpel; F, floret; G, glume; GP, glume primordium; L, lemma; LP, leaf primordium; PR, provascular strand; SM, spikelet meristem; TSM, terminal spikelet meristem.

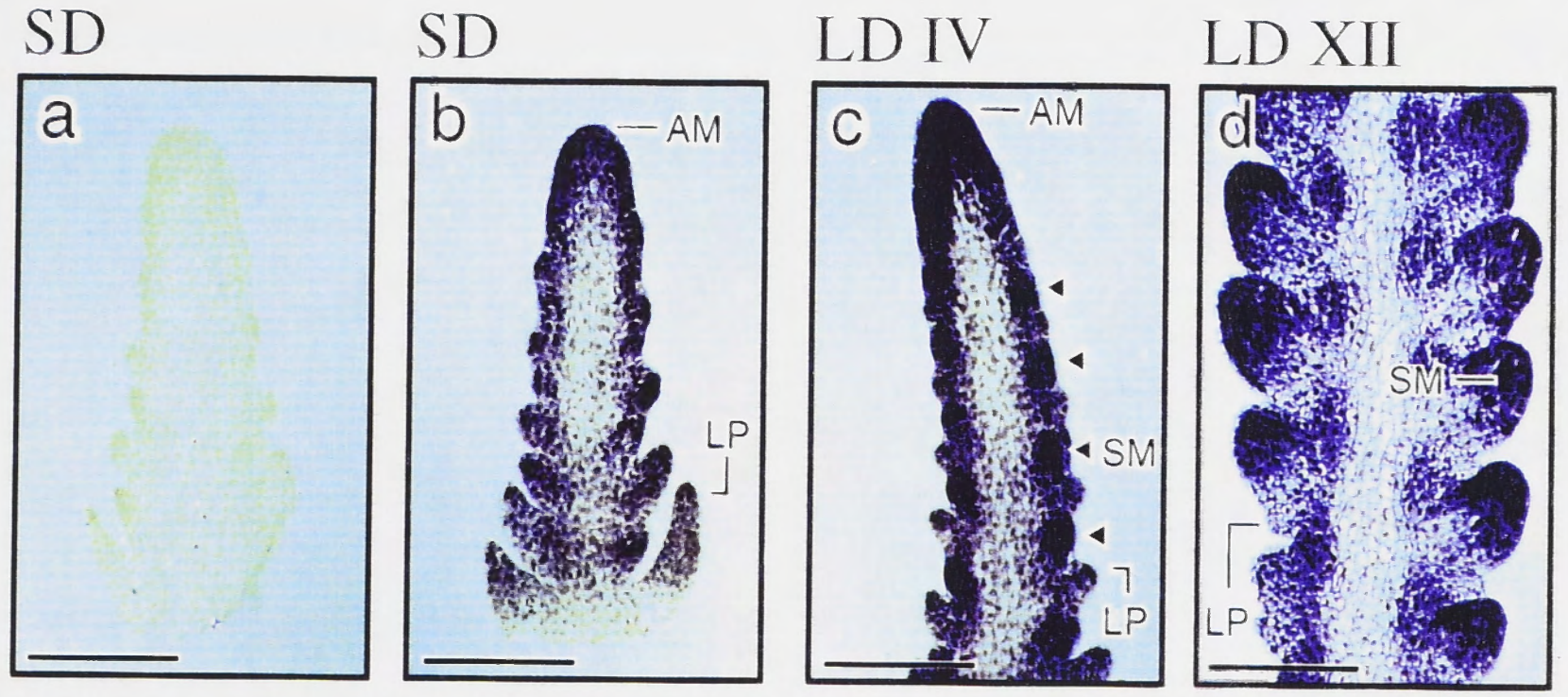
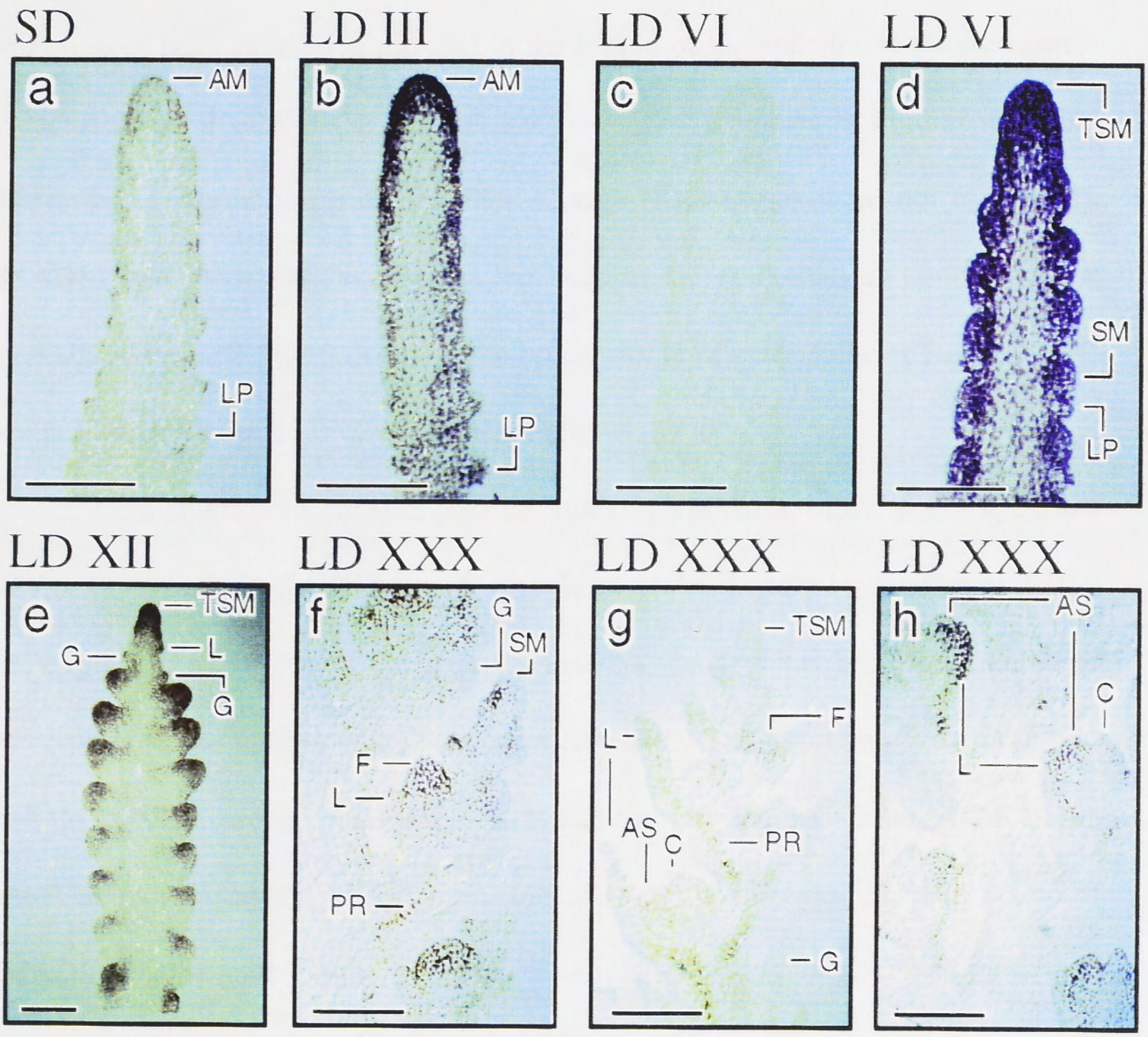

SD
a
SD
b
AM
LP
LD IV
c
AM
SM
LP
LD XII
d
SM
LP
LD XXI
e
SM
L
G
PR
LD XXX
f
TSM
L
F
G
LD XXX
g
F
L
SM
G
LD XXX
h
AS
C
L

Figure 4.10 *In* situ localisation of LtMADS2 transcripts in *Lolium* shoot apices.

In situ hybridisations were performed on longitudinal sections of wax embedded apices at different developmental stages using DIG-labelled *in vitro*-transcribed riboprobes synthesised from a gene specific fragment of the LtMADS2 cDNA (MADSr1/2; nucleotide 568 to 874). Sectioned apices were all from experiment Lt434 (see Figure 2.1). Sections (a), (b), (d), (e), (f), (g) and (h) were hybridised with the antisense riboprobe. A representative section hybridised with the sense control riboprobe is shown in (c). Hybridisations were with an equivalent sense or antisense probe concentration. The same dilution of anti-DIG antibody was used to detect riboprobe hybridisation and the duration of the colour development was identical for all sections in this figure. Hybridisation is visible as the pink to purple product of the alkaline phosphatase reaction. Photographs were taken using Nomarski optics with identical exposures. Therefore, in all antisense hybridised sections, the intensity of staining is directly proportional to the expression of LtMADS2. Median sections through (a) six-week old vegetative (SD) shoot apex, (b) LD III shoot apex 24 h after the end of the inductive LD, (c,d) double ridge shoot apices (LD VI), (e) shoot apex at glume stage (LD XII), (f) two lateral spikelet sites on the flanks of the shoot apex at floret stage (LD XXX), (g) terminal spikelet site subtended by two glume primordia with three floret meristems alternately along the terminal spikelet axis subtended by lemma primordia (LD XXX), (g) three lateral spikelet sites at floret stage (LD XXX) and (h) four floret sites with differentiated lemma, stamen and carpel primordia (LD XXX). The bars are 50 μm.

Abbreviations: AM, apical meristem; C, carpel; F, floret; G, glume; GP, glume primordium; L, lemma; LP, leaf primordium; PR, provascular strand; SM, spikelet meristem; S, stamen primordium; TSM, terminal spikelet meristem.


SD
LD III
LD VI
LD VI
a
AM
LP
b
AM
LP
c
d
TSM
SM
LP
LD XII
LD XXX
LD XXX
LD XXX
e
TSM
G
L
G
f
G
SM
F
L
PR
g
TSM
F
L
AS
C
PR
G
h
AS
C
L

of the original SD apex (see LD XII apex in Figure 4.10e). During this transition phase, expression of both genes which was initially in circular zones along the edge of the shoot meristem at the double ridge stage, became localised to the tip (especially the dorsal side) of each spikelet at the advanced double ridge stage (compare LD IV apex (c) with LD XII apex (d) of Figure 4.9 and compare LD VI apex (d) with LD XII apex (e) of Figure 4.10). Also during the transition phase, the expression of both genes was reduced in the region of the spikelet meristem nearest the rachis (ventral side of the spikelet site; see LD XII apices in Figures 4.9d and 4.10e). From lemma stage until anther + stage (later stages were not examined), LtMADS1 and LtMADS2 expression localised to the epidermal and subepidermal layers (ca. three cell layers deep) of the apical dome of each spikelet (see LD XXI apex in Figure 4.9e and LD XXX apex in Figure 4.10f).

In addition to being expressed in the apical dome of the shoot apex and spikelet sites, both LtMADS1 (see Figure 4.9e) and LtMADS2 (see Figure 4.10e) were expressed at the tips of the glume primordia (cauline leaf and floral meristem counterparts in the dicots) when they were initiated. The expression of LtMADS1 and LtMADS2, however, was only detected in the provascular strand of the sterile glumes as they elongated (see Figures 4.9f and 4.10f). Provascular expression of LtMADS1 in the core of the shoot apex was also observed at stages later than advanced double ridges (see Figure 4.9e).

Both LtMADS1 and LtMADS2 were expressed throughout the floret meristems, which initiated alternately along the axis of the spikelet (rachilla), prior to the initiation of floral organs (see LD XXX apices in Figures 4.9f and g and 4.10f). At initiation, LtMADS1 and LtMADS2 (see Figure 4.10h) transcripts were present in the lemma, stamen and carpel primordia derived from the floret meristem. Whereas,

LtMADS1 expression was retained in these primordia even at the latest stage assessed in this study (anther + stage; see LD XXX apices in Figures 4.9h), expression of LtMADS2 was initially excluded from the carpel primordia (see Figure 4.10h) and later from the stamens, but was always retained in the lemmas (see Figure 4.10g). Although both LtMADS1 and LtMADS2 appeared to be expressed in the palea primordia (located on the dorsal side of the carpel primordium), even at anther + stage, the small size of both the palea and lodicule primordia precluded adequate determination of the expression of these genes in these organs. LtMADS1 and LtMADS2 expression was also observed in progenitor cells of the vascular system of the lemma primordia. An example of LtMADS2 vascular expression extending from a lemma primordium is seen in the LD XXX apex in Figure 4.10g.

4.4 *Phylogeny of LtMADS1 and LtMADS2*

In order to determine the phylogenetic relationship of *LtMADS1* and *LtMADS2* within the plant MADS-box gene family, their protein sequences were aligned with those of many members of the SQUA branch (*SQUA*, *ZAP1*, *ZMM4* [82 amino acids compiled from Theißen *et al.*, 1996 as well as a maize EST (accession number T12733)], *SLM4* and *5*, *TM4*, *POTM1*, *SaMADS B*, *AGL8*, *CAL*, *SaMADS C*, *AP1* and *BoAP1*) and using members of the TM3 branch (*TM3*, *TobMADS1*, *SaMADS A*, *DEFH24* [partial sequence from the review of Davies and Schwarz-Sommer, 1994] and *ZMM5* [partial sequence from Theißen *et al.*, 1996]), the AG branch (*AG* and *ZAG1*), the AGL2 branch (*AGL3* and *OsMADS1*) and the TM8 branch as outliers. To provide an indication of the likely distance between monocot and dicot sequences, where available, at least one monocot sequence was included in each branch.

Sequences were first aligned using PILEUP program of the GCG package (Genetics Computer Group, Version 8, Madison, Wisconsin, USA). Four data sets were aligned (1) the MADS-box domain alone, (2) the MADS-box and I region, (3) the MADS-box, I region and K domain, and finally (4) the entire protein sequence. Partial sequences were completed with "?"s. Phylogenetic trees were constructed for each dataset using the PROTPARS program of the phylogenetic inference package (PHYLIP). As in Theißen *et al.* (1996), the input order of these sequences was randomised using the jumble option in the PROTPARS program and repeated ten times. Multiple most parsimonious trees were found in each instance and the consensus tree was determined by using the PHYLIP program CONSENSE. The consensus most parsimonious tree was drawn for each dataset using the PHYLIP program DRAWTREE.

Similar trees were observed for all four datasets. The consensus most parsimonious tree for the MADS-box dataset is presented in Figure 4.11. For each dataset, 5 branches of the MADS-box gene family (SQUA, AGL2, TM8, AG and TM3) were clearly separate from each other (labelled in capital letters on Figure 4.11). Within each branch for which both monocot and dicot sequences were available, these grouped within the expected branches. This indicates that a progenitor gene for each branch was present prior to the monocot/dicot split (Theißen *et al.*, 1996).

LtMADS1 and *LtMADS2* are clearly members of the SQUA/AP1 branch of the MADS-box gene family (see Figure 4.11). They group with their likely homologues from maize, *ZMM4* and *ZAP1* respectively, and separate from dicot members of this branch. Duplicate genes, and in the Brassicaceae three genes, are observed for many members of both the monocot and dicot lineages. Duplications in

this branch are common in both the monocot and dicot lineages and appear to have resulted independently after the monocot/dicot split since duplicate members tend to group with members of the same family (e.g., *ZMM4/ZAP1*, *LtMADSl/2*, AGL8/*AP1/CAL* and *SLM4/5*) and not across families as two separate groups (e.g., AP1 and AGL8). Since both *Lolium* and maize (and likely rice, based on available EST data) contain two members of the SQUA family, it is likely that the progenitor gene in the monocots was duplicated after the monocot/dicot split, but prior to the speciation of the cereals and grasses. For this reason, *LtMADS1*, *AGL8* and *SaMADS B* are unlikely to be true homologues. Likewise in the Brassicaceae (*Arabidopsis*, *Sinapis alba* and cauliflower), three genes are present in the SQUA branch resulting from an ancient duplication (to generate the AGL8 branch) and a more recent duplication (to generate the CAL branch; see Figure 4.11) both occurring after the monocot/dicot split.

Discussion

4.5 *Comparison of LtMADS1 and LtMADS2 with ZAP1 and other members of the SQUA branch*

The majority of plant MADS-box genes are expressed during flowering. It is therefore not surprising that two MADS-box genes increase in their expression in the shoot apical meristem of *Lolium* following LD floral induction.

4.5.1 Sequence Considerations

Both *LtMADS1* and *LtMADS2* are members of the SQUA branch of the MADS-box gene family (see Figure 4.11) and display significant similarity to other members of this branch (see Table 4.1). Over the coding region for this gene, *LtMADS2* and *ZAP1* are 88% identical at the nucleotide level and 89% identical and 94% similar at the amino acid level (see Figure 4.12). In the N-terminal (MIK

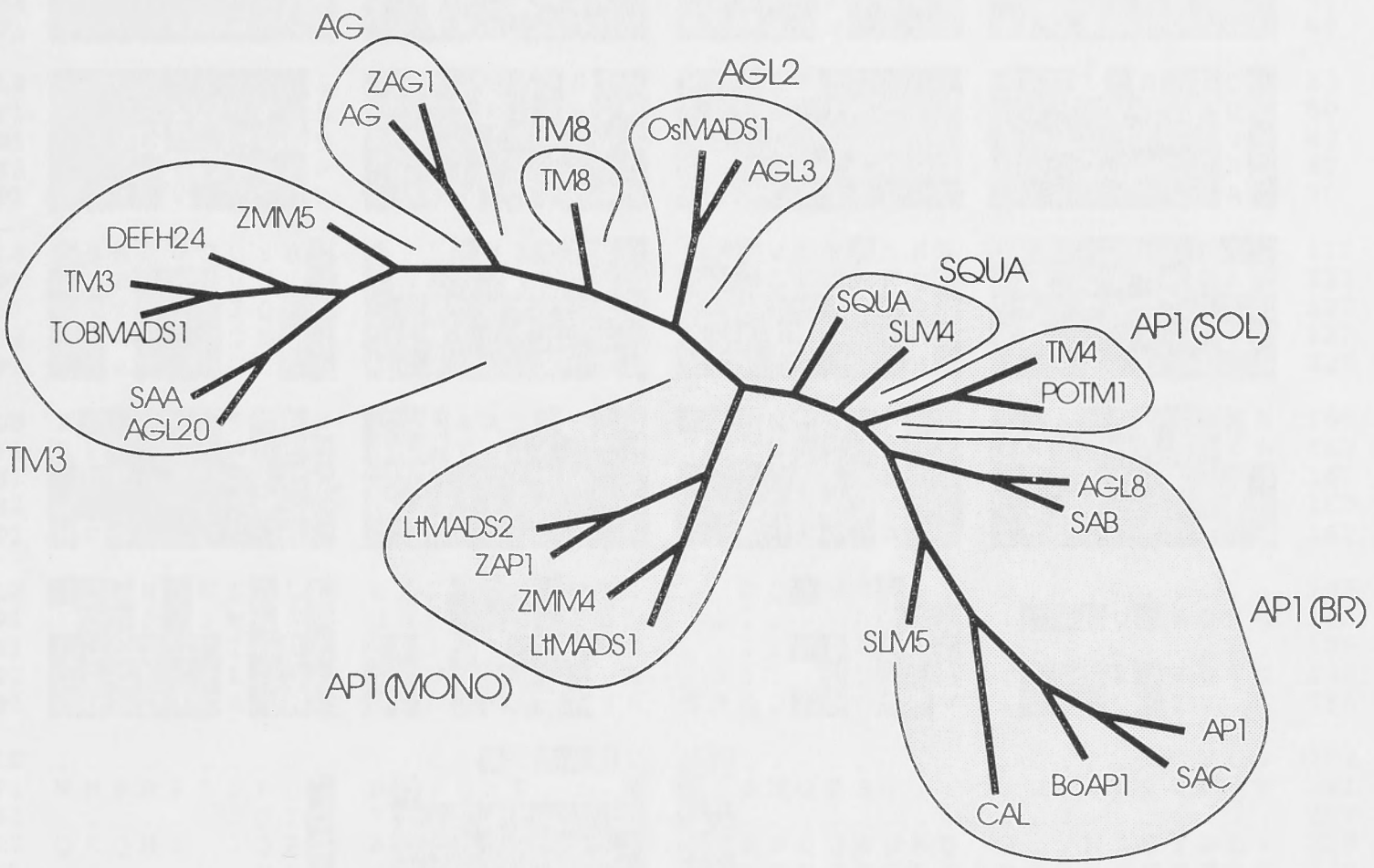


Figure 4.11 Phylogenetic tree based on complete MADS-box domain sequences of selected members of the plant MADS-box gene family.

With the exception of *LtMADS1* and *LtMADS2*, the sequences analysed and subfamily designations were as defined in Theißen *et al.* (1996). Branch lengths are proportional to the number of individual trees supporting the respective branches. Subfamilies (TM3, AG and AGL2) are encircled and named by the founding member. The SQUA subfamily (indicated with shading) is subdivided with curves indicating monocot (MONO), Brassicaceae (BR), Solanaceae (SOL) and SQUA lineages. Sequences from *Arabidopsis thaliana* (*AP1*, *CAL*, *AGL8*, *AGL3*, *AG* and *AGL20*), *Antirrhinum majus* (*SQUA* and *DEFH24*), *Brassica oleracea* (*BoAP1*), *Lolium temulentum* (*LtMADS1* and *LtMADS2*), maize (*ZAG1*, *ZAP1*, *ZMM4* and *ZMM5*), potato (*POTM1*), rice (*OsMADS1*), *Silene latifolia* (*SLM4* and *SLM5*), *Sinapis alba* (*SaMADS A* (SAA), *SaMADS B* (SAB) and *SaMADS C* (SAC)), tobacco (*TobMADS1*) and tomato (*TM3*, *TM4* and *TM8*) were analysed.

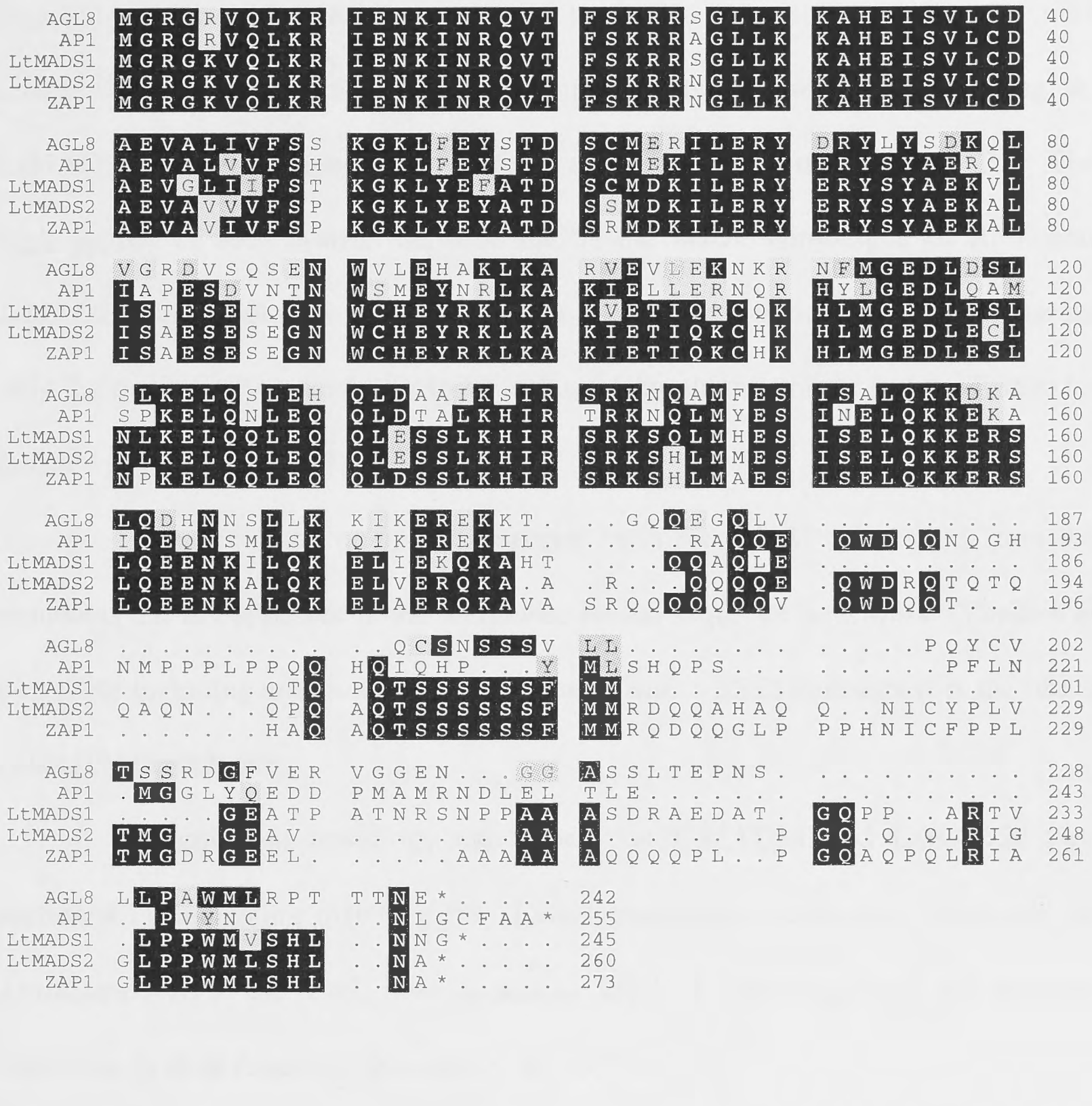


Figure 4.12 Alignment of the predicted amino acid sequences for *LtMADS1* and *LtMADS2* with several members of the SQUAMOSA branch of the plant MADS-box gene family.

Predicted amino acid sequences of *Arabidopsis thaliana AP1* (Z16421) and *AGL8* (U33473) and *Zea mays ZAP1* (L46400). Accession numbers are in brackets. Dots indicate gaps to maximise alignment. Amino acids that are identical in three or more of the proteins are blocked while those which are similar are shaded. A conserved serine (S) rich motif is indicated with bold underlining. A tryptophan (W) [amino acid 187 from LtMADS2] residue embedded in a glutamine (Q) rich region is underlined.

regions) portion of LtMADS2, compared to ZAP1, there are only 6 amino acid changes with only one of these changes being non-conservative. In this same region, LtMADS1 has 16 changes (most of them conservative) compared to ZAP1. The high degree of conservation between *ZAP1*, the maize homologue of *AP1*, and *LtMADS2* likely indicates that they are true homologues. *LtMADS2* thus represents only the second *AP1* homologue to be isolated from the monocots and the first to be isolated from a non-cereal species.

Thus far, the complete sequence for a second AP1-like gene from the monocots has not appeared in the literature. Partial sequence for *ZMM4* (Theißen *et al.*, 1996; including additional sequence from a maize EST) indicates it is the likely *LtMADS1* homologue.

Significant homology exists between LtMADS1 and LtMADS2 (see section 4.1), indicating that they may have conserved or redundant functions. In *Arabidopsis AP1* and *CAL* have a similar level of homology and are partially redundant in their functions (Kempin *et al.*, 1995).

Over the first 170 amino acids including the MADS-box, I region and K-box, members of the SQUA branch of the plant MADS-box gene family display >80% identity. Overall the C-terminus is less conserved, but displays a number of common motifs between monocot (LtMADS1, LtMADS2 and ZAP1) and dicot members of the SQUA branch. Most significant is a tryptophan (W) residue embedded in a glutamine (Q) rich region which is present in all members of this family excepting LtMADS1, AGL8 and SaMADS B (see underlined tryptophan [amino acid 187 from LtMADS2] in Figure 4.12). A conserved serine (S) rich motif is found in the monocot sequences as well as AGL8 and SaMADS B (see bold underlined serines in Figure 4.12). In LtMADS1, this sequence may represent an

Table 4.1 Comparison of *LtMADS1* and *LtMADS2* with MADS-box genes from other species.

		LtMADS1		**LtMADS2**	
Species	Clone	Nucleotide Identity	Amino Acid Sim./Ident.	Nucleotide Identity	Amino Acid Sim./Ident.
maize	ZAP1	81	88/78	88	94/89
Arabidopsis	AP1	66	84/64	66	84/68
snapdragon	SQUA	64	85/65	63	84/66
Sinapis	SaMADS C	66	90/68	65	85/67
tomato	TM4	68	87/72	65	85/68
potato	POTM1	67	86/70	65	78/67
Arabidopsis	AGL8	66	74/54	64	81/62
Sinapis	SaMADS B	66	72/53	66	86/65
Arabidopsis	AGL3	60	81/55	59	66/42

STP transcriptional activation domain (see boxed sequence in Figure 4.3). At the C-terminus of the protein, the sequence "LPPWMLSHLN" is conserved in the AP1-like monocot sequences with a similar sequence "LPAWML" found in AGL8 and SaMADS B, and the sequence "WML" found at the C-terminus of several members of the closely related AGL2 branch.

4.5.2 Expression Patterns

Conservation of amino acid sequence is accompanied by conserved patterns of mRNA expression. This study is the first to show *in situ* analysis of mRNA expression for members of the SQUA branch of the MADS-box gene family in a grass species. For this reason, expression patterns for LtMADS2 could only be compared to ZAP1 using available RNA blot data, however, the expression of both LtMADS1 and LtMADS2 were also compared with those of other dicot MADS-box genes.

The predominant expression of LtMADS2 in florally induced apices and its absence from root and leaf tissue agrees with that for ZAP1 (Mena *et al.*, 1995). By *in situ* analysis, this study has shown LtMADS2 is expressed in the apical dome, spikelet primordia and in the glume primordia at initiation, as well as in the lemma and likely the palea primordia (first whorl organs). Soon after their initiation, LtMADS2 expression was excluded initially from the carpel primordia and later from the stamens. This corresponds with the result of Mena *et al.* (1995) where, by RNA blot analysis, ZAP1 was shown to be expressed in non-reproductive organs (combined sample containing glumes, lemma, palea and lodicules) of maize male and female flowers, but was absent from their reproductive organs, stamens and carpels respectively. The ancient role of *AP1* homologues in generating the sterile

organs of florets (monocots) and flowers (dicots) might therefore be conserved throughout the angiosperms (Mena *et al.*, 1995).

The similar motifs within the C-terminus and the expression pattern of LtMADS1 is more similar to AGL8 and SaMADS B than to AP1 or SQUA. Although most published MADS-box genes are excluded from the inflorescence meristem, both LtMADS1 and LtMADS2 are expressed in the spikelet sites, similar to the inflorescence meristem expression of SaMADS A, SaMADS B and AGL8 (Mandel and Yanofsky, 1995b; Menzel *et al.*, 1996; see Table 4.2). Expression in the epidermal and subepidermal layers and exclusion from the central core of the spikelet sites enhances the similarity between LtMADS1 and AGL8 (and SaMADS B), which are excluded from the pith of the inflorescence apex (compare Figure 3 B and D from Mandel and Yanofsky, 1995b with the spikelet sites in Figures 4.9e, 4.9f and 4.9g; Menzel *et al.*, 1996). It is interesting that both LtMADS1 and LtMADS2 are expressed within the provascular strands in the central core of the meristem and in the glumes and lemmas. Provascular expression is also observed for SaMADS B (and likely the *Arabidopsis* homologue AGL8; Menzel *et al.*, 1996). Despite the obvious morphological differences and the evolutionary distance between monocots and dicots, it is interesting that the pattern of LtMADS1 expression in *Lolium* is remarkably similar to the pattern of AGL8 (*Arabidopsis*) and SaMADS B (*Sinapis*) expression.

Although LtMADS2 has some features in common with AGL8, it displays obvious similarities with AP1 such as conserved peptide motifs within their C-termini, their exclusion from the reproductive organs of the floret (flower) and their failure to express in leaf or root tissue (see Table 4.2).

Table 4.2 Summary of the expression patterns for several MADS-box genes.

		Transcripts detected in:								
							Le/Pa	**Lo**		
Species	**Clone**	**VM**	**IM**	**FM**	**L**	**R**	**Se**	**Pe**	**St**	**Ca**
Lolium	LtMADS1	+	+	+	+	+	+	?	+	+
Lolium	LtMADS2	+	+	+	-	-	+	?	+➤-	+➤-
maize	ZAP1	?	?	?	-	-	+	+	-	-
Arabidopsis	AP1	-	-	+	-	-	+	+	-	-
snapdragon	SQUA	-	?	+	-	-	+	+	-	+
Sinapis	SaMADS C	-	-	+	-	?	?	?	?	?
tomato	TM4	?	?	+	-	?	?	?	?	?
potato	POTM1	+	+	+	+	+	?	?	?	?
Arabidopsis	AGL8	-	+	+	-	+	-	-	-	+
Sinapis	SaMADS B	-	+	+	-	+	?	?	?	?
Arabidopsis	AGL3	?	?	+	+	-	?	?	?	?

VM = vegetative meristem; IM = inflorescence meristem; FM = floral meristem; L = leaf; R = root; Se = sepal; Le = lemma; Pa = palea; Pe = petal; Lo = lodicule; St = stamen; Ca = carpel.

+ indicates expression detected; - indicates expression was not detected; +➤- indicates expression detected at times proximal to organ initiation with little or no expression detected during organ development; and ? indicates expression was not determined.

* Expression patterns were summarised from RNA blot and *in situ* hybridisation experiments published for TM4 (Pnueli *et al.*, 1991), SQUA (Huijser *et al.*, 1992), AP1 (Mandel *et al.*, 1992), AGL3 (Huang *et al.*, 1995), POTM1 (Kang and Hannapel, 1995), ZAP1 (Mena *et al.*, 1995), AGL8 (Mandel and Yanofsky, 1995b), and SaMADS A, SaMADS B and SaMADS C (Menzel *et al.*, 1996).

The expression of LtMADS1 in root and leaf tissue is interesting. To date, *LtMADS1* and *POTM1*, both members of the SQUA branch as well as *TobMADS1* from the TM3 branch are the only MADS-box genes expressed in both leaves and roots (Mandel *et al.*, 1994; Kang and Hannapel, 1995). Other MADS-box genes, however, are expressed in either leaves, including *AGL3* and *AGL8* (*Arabidopsis*), *TM3* (tomato), *SaMADS A* (*Sinapis*) and *DEFH24* (*Antirrhinum*), or roots, including *AGL12*, *AGL14* and *AGL17* (*Arabidopsis*) (Davies and Schwarz-Sommer, 1994; Hareven *et al.*, 1994; Huang *et al.*, 1995; Mandel and Yanofsky, 1995b; Rounsley *et al.*, 1995; Menzel *et al.*, 1996).

Thus far, *LtMADS1* and *LtMADS2* as well as *SaMADS A* and *SaMADS B* (and likely *AGL3*) are the only MADS-box genes known to be expressed in the vegetative meristem of a mature plant prior to floral induction.

The increased expression of LtMADS1 and LtMADS2 within the dome of the shoot apex, as well as their expression in the spikelet and floret meristems indicates, in *Lolium*, these genes may generally be required for meristem identity. All MADS-box genes within the SQUA and AGL2 branches (AP1/AGL9 group in the analysis of Purugganan *et al.*, 1995), for which *in situ* hybridisation analysis has been performed, are expressed in meristematic regions within the shoot (see Table 4.2). Perhaps members of these branches were derived from a common progenitor.

LtMADS1 is also expressed in the floret meristems as well as all organs which are initiated from this meristem. Although AGL8 is only expressed in the centre of the floral meristem (stamen and carpel whorls), AGL2 from *Arabidopsis*, the founding member of the branch most closely related to the SQUA branch (see Figure 4.11), is expressed throughout the floral meristem and in all organs derived from it (Mandel and Yanofsky, 1995b; Flanagan and Ma, 1994).

Although *LtMADS1* and *LtMADS2* are 79% identical at the nucleotide level, there are obvious differences in their expression patterns. Possibly, these genes originated through duplication and subsequent divergence. Although relatively more recent, duplication, divergence and increased functional specificity also seems to have occurred for *ZAG1* and *ZMM2*, the *AG* homologues in maize, an ancient tetraploid (Theißen *et al.*, 1995). ZMM2 is expressed predominantly in the tassel, whereas ZAG1 is expressed predominantly in the ear (Mena *et al.*, 1996). Within members of each pair, divergence of their roles is apparent in sequence, in differences in expression pattern (alterations in promoter sequences) and in changes in functional specificity.

The high degree of homology between *LtMADS1* and *LtMADS2* and their uncertain relationship with respect to other members of the SQUA branch of the plant MADS-box gene family, does not allow a meaningful complementation test to determine true functional orthology to be performed. Both *LtMADS1* and *LtMADS2* might be expected to complement the *Arabidopsis ap1* mutant phenotype to a certain degree. If LtMADS1 and LtMADS2 were overexpressed in *Arabidopsis* under the control of the constitutive 35S promoter they might be expected to cause a dominant gain of function *terminal flower* phenotype, as does overexpression of AP1 (Mandel and Yanofsky, 1995b). A meaningful functional analysis of *LtMADS1* and *LtMADS2* would best be performed by transforming these genes into a homologous grass (preferably *Lolium*) or cereal system under the control of the 35Sp in both sense and antisense orientations.

4.6 *LtMADS1 and LtMADS2 and Flowering*

Aside from being specifically induced to flower with a single LD, characterisation of the *Lolium* system over the past 40 years offers a wealth of

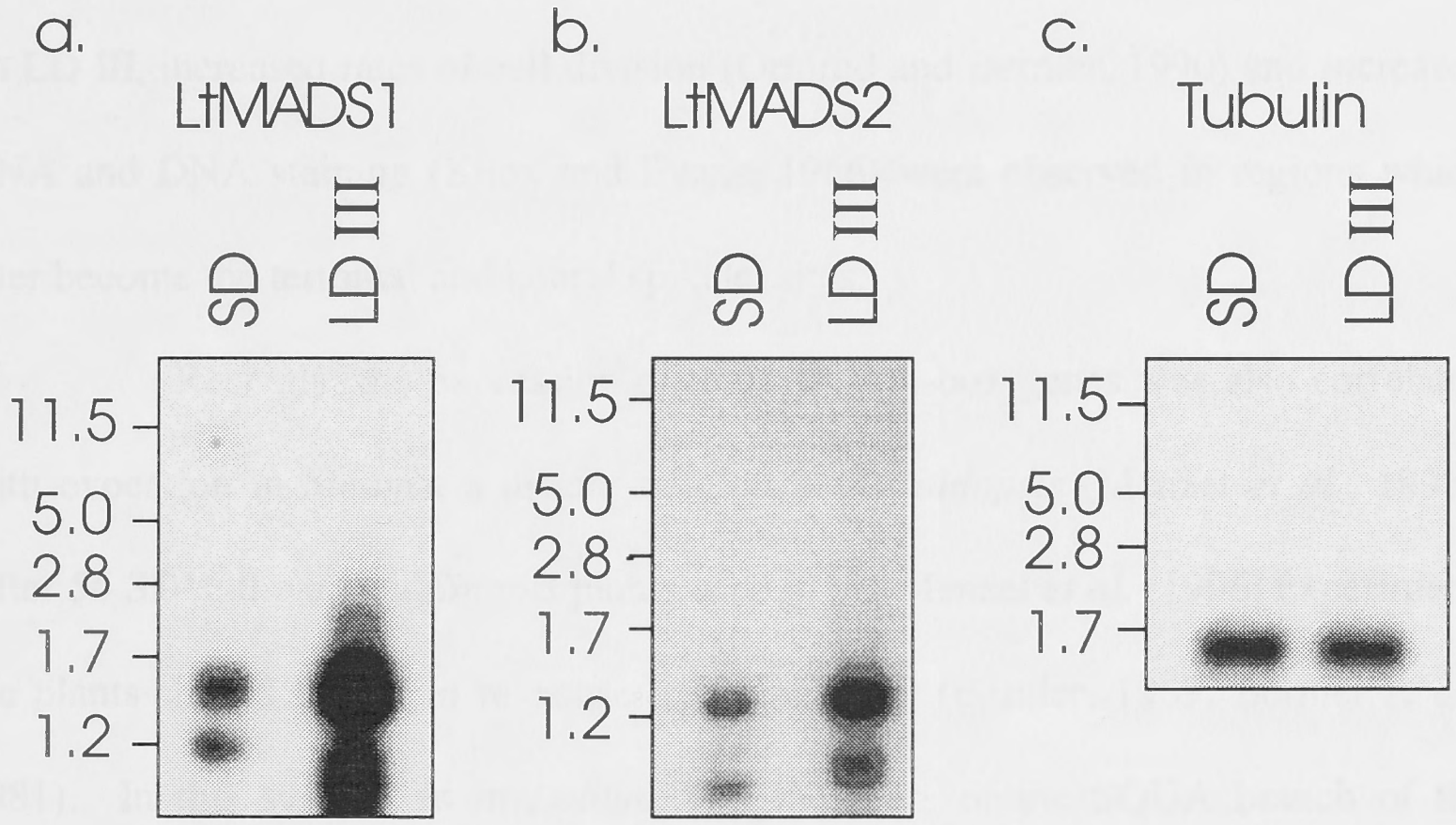


Figure 4.13 Virtual northern blot analysis of vegetative SD and florally induced LD III (24 h after the end of the single LD) PCR-based cDNA hybridised with (a) a gene specific probe for *LtMADS1* (MADSc1/2; nucleotide 650 to 1062), (b) a gene specific probe for *LtMADS2* (MADSr1/2; nucleotide 568 to 874) or (c) a fragment of a *Lolium* tubulin cDNA (see Table 2.3.4) to compare loading between lanes. Sizes in kbp are indicated to the left.

physiological information. Synthesising this physiological data with the molecular data obtained in this study, it is immediately apparent that the increased expression of LtMADS1 and LtMADS2, evident by the afternoon of LD III (the day after the long day; see Figure 4.13), correlates with commitment of the shoot apical meristem to flower assessed by culturing induced apices *in vitro* (McDaniel *et al.*, 1991). Also on LD III, increased rates of cell division (Ormrod and Bernier, 1990) and increased RNA and DNA staining (Knox and Evans, 1966) were observed in regions which later become the terminal and lateral spikelet sites.

Recently, the expression of three MADS-box genes was also correlated with evocation in *Sinapis*, a distant relation of *Arabidopsis* (Menzel *et al.*, 1996). After 56 SD's, the age of *Sinapis* plants used in the Menzel *et al.* (1996) experiment, the plants should flower in response to a single LD (Bernier, 1969; Bernier *et al.*, 1981). In this system, as in *Lolium*, two members of the SQUA branch of the MADS-box gene family were highly up-regulated within a few days following LD floral induction (specifically SaMADS B and SaMADS C; Menzel *et al.*, 1996). Unlike *Lolium*, however, the first MADS-box gene to be up-regulated was *SaMADS A*, a member of the TM3 branch (Menzel *et al.*, 1996). In both systems, up-regulation of MADS-box genes is correlated with commitment to flower (Bernier, 1976; McDaniel *et al.*, 1991).

4.7 *Cell Division and LtMADS1 and LtMADS2 Expression*

All known members of the SQUA branch (and likely those of the closely related AGL2 branch) are expressed meristematically (vegetative, inflorescence and floral) (see Table 4.2). They might therefore be involved in activating/specifying meristems of various types both during the floral transition and thereafter. However, their expression in vegetative, inflorescence and floral organ primordia (see Table

4.2) and in provascular cells suggests their role may be even more basic as factors which increase the level of cell division in localised regions of the plant.

In *Arabidopsis*, ectopic expression of AGL8 in the young floral primordia of *ap1* mutant flowers correlates both with the appearance of secondary flowers in the axils of the sepals (an inflorescence character) as well as with ectopic cell division in that region (Mandel and Yanofsky, 1995b). Two MADS-box genes responsible for petal and stamen identity in *Arabidopsis*, *AP3* and *PI*, have also been correlated with initiating and maintaining cell division (Weigel and Clark, 1996). Mutation of *AP3* and *PI* result in a reduced stamen number (Bowman *et al.*, 1991; Jack *et al.*, 1992), whereas, either co-overexpression under the control of the viral 35S promoter, or endogenous overexpression of these genes as a result of the *superman* mutation, an antagonist of AP3 and PI expression, resulted in the formation of additional whorls of stamens at the centre of the flower (Jack *et al.*, 1994; Krizek and Meyerowitz, 1996). Finally, when AP3 and PI are co-overexpressed under the control of 35S promoter in a *superman* mutant background, the resulting flowers become indeterminate (Krizek and Meyerowitz, 1996). Supporting this correlation, Schwarz-Sommer *et al.* (1992) found that the expression of CDC2a and CDC2b overlapped with the expression of the B class floral homeotic gene *DEF*. The expression of CDC2 and that for MADS-box genes is thus closely tied in both *Antirrhinum* and *Lolium*. However, in *Lolium* this link has been demonstrated for members of the SQUA family as opposed to the DEF/GLO family. *OsMADS1* also appears to have a role in controlling cell division, since constitutive overexpression of this gene in tobacco plants results in the proliferation of axillary buds (loss of apical dominance) causing a more branched phenotype (Chung *et al.*, 1994).

Cell division and the expression of LD up-regulated MADS-box genes is also correlated in both time and space during evocation for *Lolium* and *Sinapis*, both LD plants. In *Lolium*, LtMADS1, LtMADS2 and CDC2aLt co-express following LD floral induction (compare Figures 4.9 and 4.10 with Figure 3.5, respectively). Whereas in *Sinapis*, increased expression of SaMADS A and SaMADS B, in the peripheral zone of the apical meristem, is observed after two LD's just following the first wave of new cell division, as opposed to G2 release (62 h after induction) within the same region of the apex (Bernier, 1969; Menzel *et al.*, 1996). In both species, commitment to flower is correlated with the occurrence of both events.

4.8 *Differential Splicing of LtMADS2*

The position of intron 4 within *AP1*, *SQUA* and *LtMADS2* is conserved (Huijser *et al.*, 1992; Mandel *et al.*, 1992). In addition to the high degree of homology within these genes (see Table 4.1) and the similarity with respect to their *in situ* expression patterns, their conserved intron positioning is also supportive of *LtMADS2*'s homology with *AP1*.

Differential splicing has been observed in MADS-box genes in both plants and animals. In maize, five alternatively spliced mRNA products are derived from the ZEMa pre-mRNA with splice site preference varying between tissues (Montag *et al.*, 1995). Differential splicing of the AP1 transcript also occurs in eucalyptus. In this case, a 38 nucleotide fragment is retained from intron 7 resulting in a frame shift and premature termination of translation (Kyozuka, personal communication). It is interesting that in both eucalyptus and *Lolium* a truncated protein would result from alternative splicing of a member of the SQUA branch. Perhaps AP1 proteins (and MADS-box proteins in general) lacking their C-terminal transactivation domain play an important post transcriptional role in other species?

The only evidence suggesting translation of differentially processed MADS-box genes into proteins comes from an immunoblot of soluble protein extracts from several species of immature seeds probed with affinity-purified antibody against the K-domain of AGL15. For papaya, protein species of 29 and 18.4 kda were detected (Heck *et al.*, 1995), with the smaller species being about the expected size for a protein truncated after the K-box. *AGL15* genes are structurally most similar to *AP1* genes with their MADS-box and K-box domains having highest amino acid identity with AP1 (Heck *et al.*, 1995).

Assuming the truncated LtMADS2' protein is produced efficiently, it is interesting to speculate on its role. Given the fact that this protein would contain a complete N-terminus (MADS-box, I region and K-box), it should dimerise with other appropriate MADS-box partners and therefore bind DNA (Krizek and Meyerowitz, 1996). Knowing this, two possible roles come to mind. If the C-terminus of this protein is unimportant, the function of the truncated product might not be different from that of the full length form. Support for this, comes from the fact that when the N-terminus of AP1 (MADS-box and I region) was fused to the C-terminus (K-box and C region) of AG and ectopically overexpressed in *Arabidopsis*, the overexpression phenotype was indistinguishable from that resulting from ectopic overexpression of the native *AP1* gene. This however, was not a truncated product, but rather a hybrid MADS-box gene and one must not dismiss that although the sequence similarity within the C-terminal domain of AP1 and AG is low, they might share three dimensional tertiary structure or display functional similarity. A second possibility is that the C-terminal domain is important in regulating this protein's activity. Taking into account that MADS-box proteins act as dimers (homo or hetero), the truncated protein might serve as a competitor with full length proteins to

cause the formation of heterodimers with altered levels of activity (inactive or constitutively active). Support for such a role comes from studies of the *ag*-like mutant phenotype of wild type *Arabidopsis* plants transformed with a 35Sp:AG lacking its C-terminus (Mizukami *et al.*, 1996). In this experiment the construct containing the MADS-box/I region/K-box domains displayed a phenotype consistent with it having the greatest ability to act as a dominant negative regulator (Mizukami *et al.*, 1996). These two hypotheses could be tested by comparing the phenotype of plants transformed with the full length LtMADS2 transcript with that of plants transformed with a LtMADS2' transcript which retains the 20 bp intron fragment so generating a putative truncated protein.

Differential splicing is common for introns within MADS-box genes from animals and has been shown to result in multiple transcript sizes from the same pre-mRNA. For example, in cardiac muscle development, differential splicing of three MADS-box genes *MefA*, *B* and *C* could result in over 100 permutations and combinations of their protein products (Olson *et al.*, 1996). As is the case for LtMADS2, these cardiac MADS-box genes are differentially spliced C-terminally to the DNA binding region of the protein, within the transcriptional activation domain. In *Lolium*, the lack of the transcriptional activation domain could alter the activity of the translated protein.

Although transposon-induced alternative splicing of pre-mRNAs has been reported for several maize mutants, only a few examples of alternative splicing of native plant genes have appeared in the literature (Luehrsen and Walbot, 1994). Interestingly, most reports have been for transcriptional regulators. These include multiple transcripts from three families of transcription factors, a rice *HD-ZIP* gene, a maize *MYB* gene and a maize *MYC* gene (Grotewold *et al.*, 1991; Tamaoki *et al.*,

1995; Burr *et al.*, 1996). The rice *OSH45* gene is a *HD-ZIP* gene which produces 3 alternatively spliced transcripts (Tamaoki *et al.*, 1995). Here, the DNA binding domain is found in all transcripts with the alternative protein products having different transactivation activities (Tamaoki *et al.*, 1995). The maize *P* gene, a MYB responsible for regulation of flavonoid biosynthesis in certain floral tissues, produces two alternatively spliced mRNAs (Grotewold *et al.*, 1991). Here, the truncated protein would be predicted to bind DNA by virtue of the retained R2 and R3 repeats, but would not be capable of transactivation since these domains are present within the C-terminus of the protein. In a recent report multiple transcripts were shown to originate from the *INTENSIFIER1* gene, a MYC transcription factor from maize (Burr *et al.*, 1996). Like intron 4 of *LtMADS2*, two introns within the *INTENSIFIER1* gene each contain multiple 5' donor and a single 3' acceptor site. *In vivo* translation of the differentially spliced mRNAs for any of the above genes has not been demonstrated.

Although mRNA for *LtMADS1* and *LtMADS2* genes is expressed in many cells throughout the apex this does not ensure their translation into protein, nor that these proteins are present in the same cells in which they are translated, or even that they are imported into the nucleus thereby acting as transcriptional regulators. Immunolocalisation of LtMADS1, LtMADS2 and LtMADS2' should therefore be performed. A precedent for translational repression of MADS-box genes comes again from muscle development where MEF2A mRNA is present in vascular smooth muscle cells, without any accompanying protein (Suzuki *et al.*, 1995). It is also possible that their protein products may be more widely distributed than the original transcripts. Although KNOTTED-1 was the first example of mRNA/protein for a regulatory gene being transported across cell layers in a plant system (Jackson *et al.*,

1994), MADS-box proteins can also be transported. In L2L3 periclinal chimeras of DEFICIENS, its protein is detected in the L1 layer, with mRNA for this gene only expressed in L2 and L3 cells (Perbal *et al.*, 1996). Finally, there appears to be regulation at the level of entry into the nucleus. When AGL15 expression was assessed immunohistochemically, the protein was shown to be present within the egg apparatus and in the zygote early after fertilisation, however, this protein was primarily localised in the cytoplasm of the egg apparatus and did not become nuclearly localised until several days post fertilisation (Perry *et al.*, 1996).

The story increases in complexity again with superimposed on such transcriptional regulation, the possibility for regulation of protein activity in the form of phosphorylation. The predicted LtMADS1 and LtMADS2 proteins have several potential sites (R/KXXS/T) for calmodulin-dependent phosphorylation (see Figure 4.3 and 4.4; sites boxed; Cohen, 1988). The RQVT site which is phosphorylated by this type of kinase is present within the MADS-box domain and is conserved in all MADS-box genes (Davies and Schwarz-Sommer, 1994). Although calmodulin-dependent protein kinases have not been found in plants, calcium-dependent protein kinases are present and phosphorylate the same sites (Harper *et al.*, 1991; Roberts and Harmon, 1992). Phosphorylation sites for casein kinase II ($S/TX_{0-2}E/D$), another plant kinase, also exist within the predicted LtMADS1 and LtMADS2 proteins (see Figure 4.3 and 4.4; sites underlined; Kennelly and Krebs, 1991). Phosphorylation of SRF has been shown to increase its activity, measured as DNA binding affinity for the serum response element (Janknecht *et al.*, 1992), and might similarly increase the activity of LtMADS1 and LtMADS2.

Conclusions

Within 24 h of the end of the LD, the expression of LtMADS1 and LtMADS2, both MADS-box transcription factors, increased in the shoot apical meristem of *Lolium* . Both genes are members of the SQUA branch of the MADS-box gene family. The maize homologues of *LtMADS1* and *LtMADS2* are most likely *ZMM4* and *ZAP1*, respectively.

In both *Lolium* and *Sinapis*, two members of the SQUA family are up-regulated following LD floral induction. In *Lolium* LtMADS1 and LtMADS2 are expressed prior to floral induction, whereas in *Sinapis* neither SaMADS B nor SaMADS C is expressed prior to floral induction. Therefore, although LtMADS1 and LtMADS2 are likely important in the transition of the apex to flower in *Lolium*, the *in situ* hybridisation experiments indicate that the expression of these genes is not unique or novel to floral evocation.

The expression pattern of LtMADS1 and LtMADS2 is not identical to any of the published members of the SQUA branch. Overall, the expression pattern for AGL8 is most similar to that of LtMADS1. Both genes are expressed in an epidermal/subepidermal cone around the edge of the apex and both genes are expressed in the inflorescence (spikelet) meristem. LtMADS1 is distinct from AGL8 in that it is expressed in all organs derived from the floret meristem (expression in palea and lodicule primordia is unclear) whereas AGL8 is excluded from the sepal and petal primordia. LtMADS1 is also distinct from AGL8 since it is also expressed in both leaf and root tissue whereas AGL8 is expressed only in leaf tissue. The expression of LtMADS1 is similar to that of AGL2 since both genes are expressed within the floral meristem and all floral organs. AGL2 is not, however, expressed in vegetative or inflorescence meristems.

The expression pattern of LtMADS2 is more typical of that seen for members of the SQUA branch. Firstly, it is not expressed in either leaf or root tissue which agreed with the results obtained for ZAP1. Extending the ZAP1 results, in *Lolium in situ* hybridisation showed that LtMADS2 was expressed in the vegetative, spikelet (inflorescence) and floret (floral) meristems. Later in development, LtMADS1 continued to be expressed in the lemma primordia, but was eventually restricted from the carpel primordia and stamens. LtMADS2, ZAP1, AP1 and SQUA are thus all expressed in floret (floral) meristems as well as sterile organs of the perianth.

Finally, LtMADS2 pre-mRNA is differentially processed resulting in two putative protein products: (1) a full length protein, and (2) a protein which is truncated following the K-box (at amino acid 153). This truncated protein may be involved in postranscriptional regulation of LtMADS2 activity. Further progress in the understanding the *in planta* function of these MADS-box genes will arise from studying the translational regulation of their protein products as well as the multitude of post translational regulations which may influence their activities.

CHAPTER 5

LATE INCREASES IN *LOLIUM* LEAFY EXPRESSION FOLLOWING LONG DAY FLORAL INDUCTION

Introduction

The transition to flowering involves activation of a number of specific flowering-associated genes. The expression of two genes *FLORICAULA* (*FLO*) and *SQUAMOSA* (*SQUA*) from *Antirrhinum* and their cognate homologues *LEAFY* and *APETALA1* from *Arabidopsis* increases early during floral development (Coen *et al.*, 1990; Huijser *et al.*, 1992; Mandel *et al.*, 1992; Weigel *et al.*, 1992) These genes are essential for flowering since mutants of *leafy* display (i) a spiral rather than whorled arrangement of "floral" organs, (ii) visible internodes between successive organs within a whorl, (iii) leaf-like floral organs, and (iv) indeterminate inflorescence meristems where determinate floral meristems would normally form (Weigel and Meyerowitz, 1993). By contrast, *ap1* mutants display bract-like first whorl organs with secondary (and higher order) floral buds in their axils (Irish and Sussex, 1990). Ectopic overexpression of LEAFY or AP1 under the control of the 35S promoter producing a *terminal flower* mutant phenocopy, also supports their essential role in flowering (Mandel and Yanofsky, 1995a; Weigel and Nilsson, 1995). Together, *LEAFY* and *AP1* act to specify floral meristem identity (Huala and Sussex, 1992; Weigel *et al.*, 1992). Even so, the *leafy* mutant seems to display more vegetative characters implying it acts at an earlier stage or is more critical for the initiation of floral meristems. For this reason, cloning of the *Lolium LEAFY* homologue was pursued for use as a molecular indicator of commitment to floral development.

LEAFY, and its homologues from other species, represent a unique gene, encoding a protein which has features such as a proline-rich N-terminus and a highly acidic central region, both characteristics of transcription factors (Weigel and Meyerowitz, 1993). Full length cDNA's for LEAFY (Weigel *et al.*, 1992) and its homologues from *Antirrhinum* (*FLO*; Coen *et al.*, 1990), cauliflower (Anthony *et al.*, 1993) and tobacco (*NFL1* and *NFL2*; Kelly *et al.*, 1995) have been isolated. Additionally, gene fragments encoding peptides of 25 amino acids within the conserved central region of *LEAFY* have been isolated by PCR from species as diverse as corn and *Ginkgo*, a gymnosperm (Weigel and Meyerowitz, 1993).

Through the cloning of the *LEAFY* homologue from *Lolium* I have been able to define its temporal and (ii) spatial expression pattern following LD floral induction with the aim of using it as a floral specific marker. Additionally, by cloning a monocot homologue for this gene, the timing and spatial localisation of its expression could be compared with those of the cognate dicot homologues.

Results

5.1 *Cloning of the LEAFY Homologue from Lolium*

The *LEAFY*, *FLORICAULA* and *NFL* nucleotide sequences from *Arabidopsis*, *Antirrhinum* and tobacco, respectively, were aligned and the degenerate primers Glfy1 [5'-d(CG**GAATTC**ATGCGVCACTACGTG/TCACTG)-3'] and Glfy2 [5'-d(CG**GGATCC**ACATACCARATVGMGAGRCGVGG)-3'] designed to amplify a 206 bp fragment corresponding to amino acids 310 to 377 from exon 3 of LEAFY. This fragment was amplified using the PCR reaction mix and cycling conditions detailed in section 2.3.1 with an annealing temperature of 53°C throughout all 30 cycles. Amplification of this fragment has been achieved from

a.

```
  1 ATGCGCCACTACGTGCACTGCTACGCGCTGCACTGCCTCGACCAGGAGGCCTCCGACGCG  60
  1  M  R  H  Y  V  H  C  Y  A  L  H  C  L  D  Q  E  A  S  D  A   20

 61 CTGCGCCGCGCGTACAAGGCCCGCGGCGAGAACGTCGGCGCCTGGAGGCAGGCATGCTAC 120
 21  L  R  R  A  Y  K  A  R  G  E  N  V  G  A  W  R  Q  A  C  Y   40

121 GCGCCGCTCGTCGACATCGCCGCCGGCCACGGCTTCGACGTCGACGCCGTCTTCGCCGCG 180
 41  A  P  L  V  D  I  A  A  G  H  G  F  D  V  D  A  V  F  A  A   60

181  CACCCTCGTCTCTCTATTTGGTATGT 206
 61   H  P  R  L  S  I  W  Y     68
```

b.

Species	Clone	Acc. No.	Nucleotide Identity	Amino Acid Identity	Amino Acid Similarity
A.m.	FLORICAULA	M55525	74	79	88
A.t.	LEAFY	M91208	73	82	92
N.t.	NFL1(2)	U16172(4)	74	79	89
B.o.	BOFH	Z18362	71	82	91
P.r.	PrLFY	U67868	N.A.	83*	85*

* comparison over 42 amino acids

A.m. is *Antirrhinum majus*; *A.t.* is *Arabidopsis thaliana*; *N.t.* is *Nicotiana tabacum*; *B.o.* is *Brassica oleracea*; *P.r.* is *Pinus radiata*.

Figure 5.1 (a) Nucleotide and translated amino acid sequence of LtLFY1/2.

(b) Homology of LtLFY1/2 with equivalent fragments of published LEAFY homologues.

genomic DNA as well as from vegetative SD and florally induced LD III and LD XXX cDNA (data not shown). This fragment was gel purified, its ends digested with EcoR I and BamH I (restriction endonuclease sites indicated in bold above) and cloned into pBluescript SK(+) plasmid vector which had been digested with the same restriction endonucleases. Six cloned PCR-fragments were sequenced and determined to represent a single species (see Figure 5.1a) with very high homology with LEAFY homologues from other species (see Figure 5.1b). Therefore the fragment was termed LtLFY1/2.

5.2 *Expression of the Lolium LEAFY Homologue*

For *in situ* mRNA analysis of LtLFY message, the LtLFY1/2 EcoR I/BamH I fragment was recloned into pBluescript KS(+) thereby producing a construct from which template for synthesis of the sense riboprobe could be made. The LtLFY1/2 fragment hybridised to a single band in each lane of a *Lolium* genomic DNA blot (see Figure 5.2). Both sense and antisense DIG-labelled riboprobes were synthesised from the T7 promoter. Colorimetric detection of the hybridising riboprobes was via an anti-DIG Fab fragment conjugated to alkaline phosphatase.

No staining was detectable in sections of the *Lolium* shoot apex hybridised with the LtLFY1/2 sense control probe. A representative section of a LD XII shoot apex hybridised with the sense control riboprobe is shown in Figure 5.3e. At the same stage (LD XII), hybridisation of the antisense probe with LtLFY mRNA is well above that for the sense probe (see Figures 5.3b, 5.3f and 5.3g). This was the only stage at which LtLFY RNA expression was detectable (see Figure 5.3b), therefore expression of LtLFY mRNA is transient. At this time, LtLFY was detected throughout the dome of the shoot apex as well as at the tips of glume and throughout

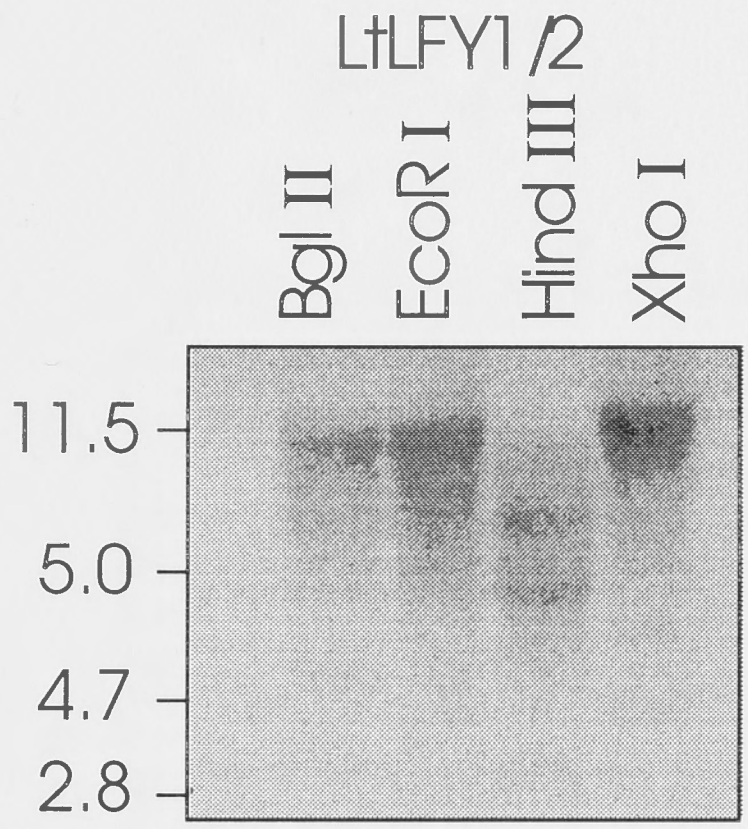


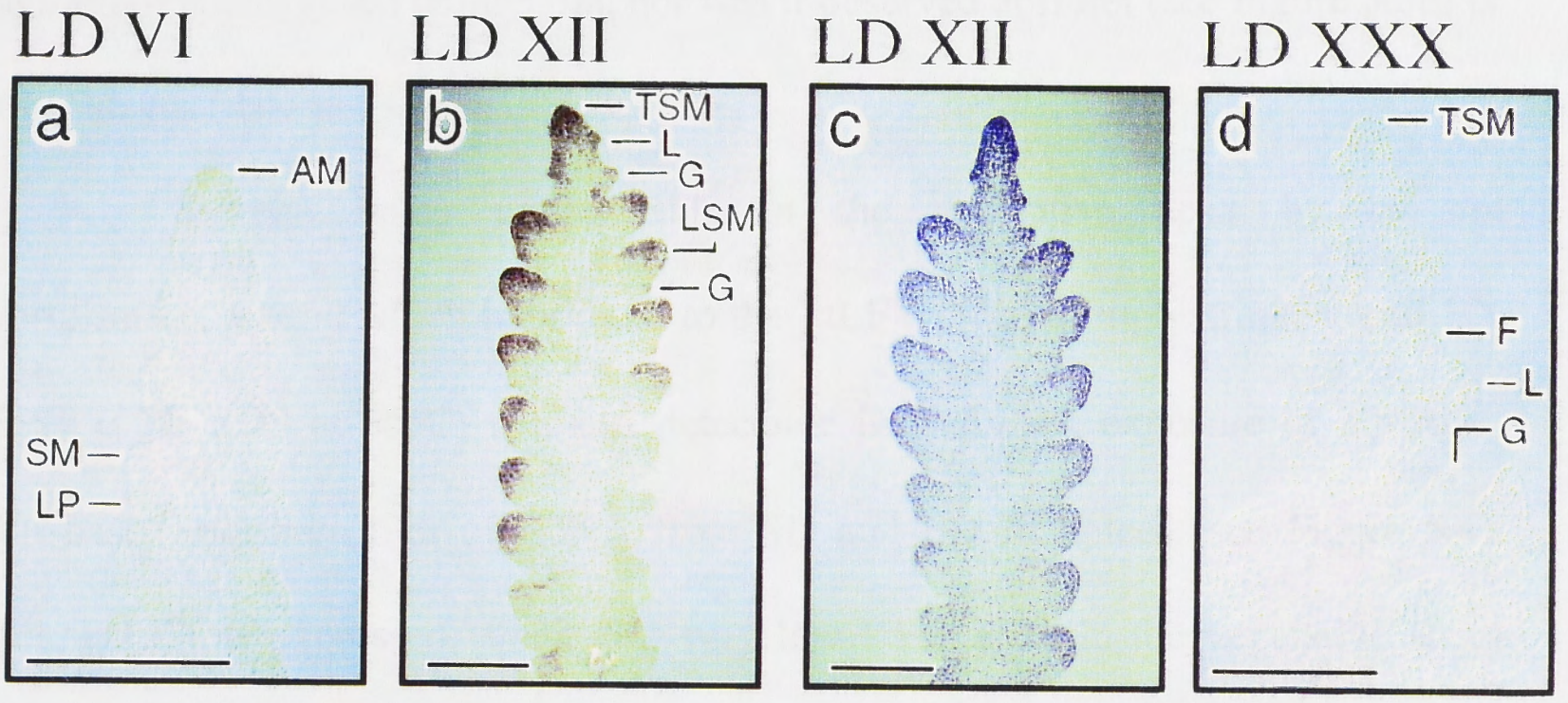
Figure 5.2 *Lolium* genomic DNA gel blot analysis with LtLFY1/2 probe.

Genomic DNA (20 μg) was digested with Bgl II, EcoR I, Hind III and Xho I. After blotting, the digested DNA was hybridised with a fragment of the *LtLFY* gene within exon 3 (LtLFY1/2). Sizes in kbp are indicated to the left.

Figure 5.3 *In* situ localisation of LtLFY transcripts and immunolocalisation of LtLFY protein in *Lolium* shoot apices.

In situ hybridisations were performed on longitudinal sections of wax embedded apices at glume stage (LD XII) using DIG-labelled *in vitro*-transcribed riboprobes synthesised from a fragment of the *LtLFY* gene within exon 3 (LtLFY1/2). All sectioned shoot apices were from experiment Lt434 (see Figure 2.1). Sections (a), (b), (d), (f) and (g) were hybridised with the antisense riboprobe. A representative section hybridised with the sense control riboprobe is shown in (e). Hybridisations were with an equivalent sense or antisense probe concentration. The same dilution of anti-DIG antibody was used to detect riboprobe hybridisation and the duration of the colour development was identical for sections (a), (b), (d), (e), (f) and (g). Section (c) was incubated with *Arabidopsis* anti-LEAFY antibody and staining with a secondary goat anti-rabbit affinity-purified antibody conjugated to alkaline phosphatase (GIBCO-BRL). Signal is visible as the pink to purple product of the alkaline phosphatase reaction. Photographs were taken using Nomarski optics with identical exposures. Median sections through (a) a double ridge shoot apex (LD VI), (b, c, e) shoot apices at glume stage (LD XII), (d) terminal spikelet site subtended by two glume primordia with floret meristems initiating alternately along the terminal spikelet axis subtended by lemma primordia (LD XXX), (f) the terminal spikelet meristem with two glume (base) and three lemma (indicated with arrow heads) primordia initiating on its flanks, and (g) three lateral spikelet sites near the base of a glume stage shoot apex. The bars are 50 μm.

Abbreviations: F, floret; G, glume; L, lemma; LP, leaf primordium; PR, provascular strand; AM, apical meristem; SM, spikelet meristem; LSM, lateral spikelet meristem; TSM, terminal spikelet meristem.


LD VI
a
AM
SM
LP
LD XII
b
TSM
L
G
LSM
G
LD XII
c
LD XXX
d
TSM
F
L
G
LD XII
e
LD XII
f
TSM
L
G
G
LD XII
g
SM
PR
G

initiating lemma primordia (see Figure 5.3f). Similar to CDC2aLt, LtMADS1 and LtMADS2 expression in glume/lemma stage apices, LtLFY expression was concentrated in the top half of the lateral spikelet meristems (see Figure 5.3b and 5.3g). Expression was not detectable in vegetative, pre-double ridge or double ridge stage shoot apices (see Figure 5.3a), nor was it observed at floret (see Figure 5.3d) or anther stages (data not shown).

Despite being undetectable in the vegetative apex by *in situ* hybridisation, a band which hybridised to the LtLFY probe at high stringency (0.1 X SSPE; 0.2% SDS at 65°C) was just detectable in a 2 week exposure of a virtual, PCR-based, northern blot of cDNA from SD and LD III apices (see Figure 5.4). Thus, LtLFY is expressed, albeit at a very low level, even in the vegetative shoot apex. This finding confirms the result that the Glfy1/2 primer pair could amplify a fragment from LtLFY in SD and LD III apices (see section 5.1). The detection of a specific band in each of the SD and LD III LtLFY1/2-probed material makes it unlikely (but not impossible) that the LtLFY1/2 fragment was amplified from contaminating genomic DNA in this material, especially since the cDNA in these pools is produced from poly (A)+ RNA. Likewise, amplification of this fragment from LD XXX cDNA material (floret/anther stage apices; data not shown) indicates LtLFY is also expressed at late stages of development, but is undetectable by *in situ* hybridisation (see Figure 5.3d).

5.3 *LEAFY Immunolocalisation*

A polyclonal anti-LEAFY antibody (generous gift of Dr E.M. Meyerowitz, California Institute of Technology, Pasadena, CA, USA) was used to examine the expression of LtLFY on paraffin-embedded formalin-fixed apices

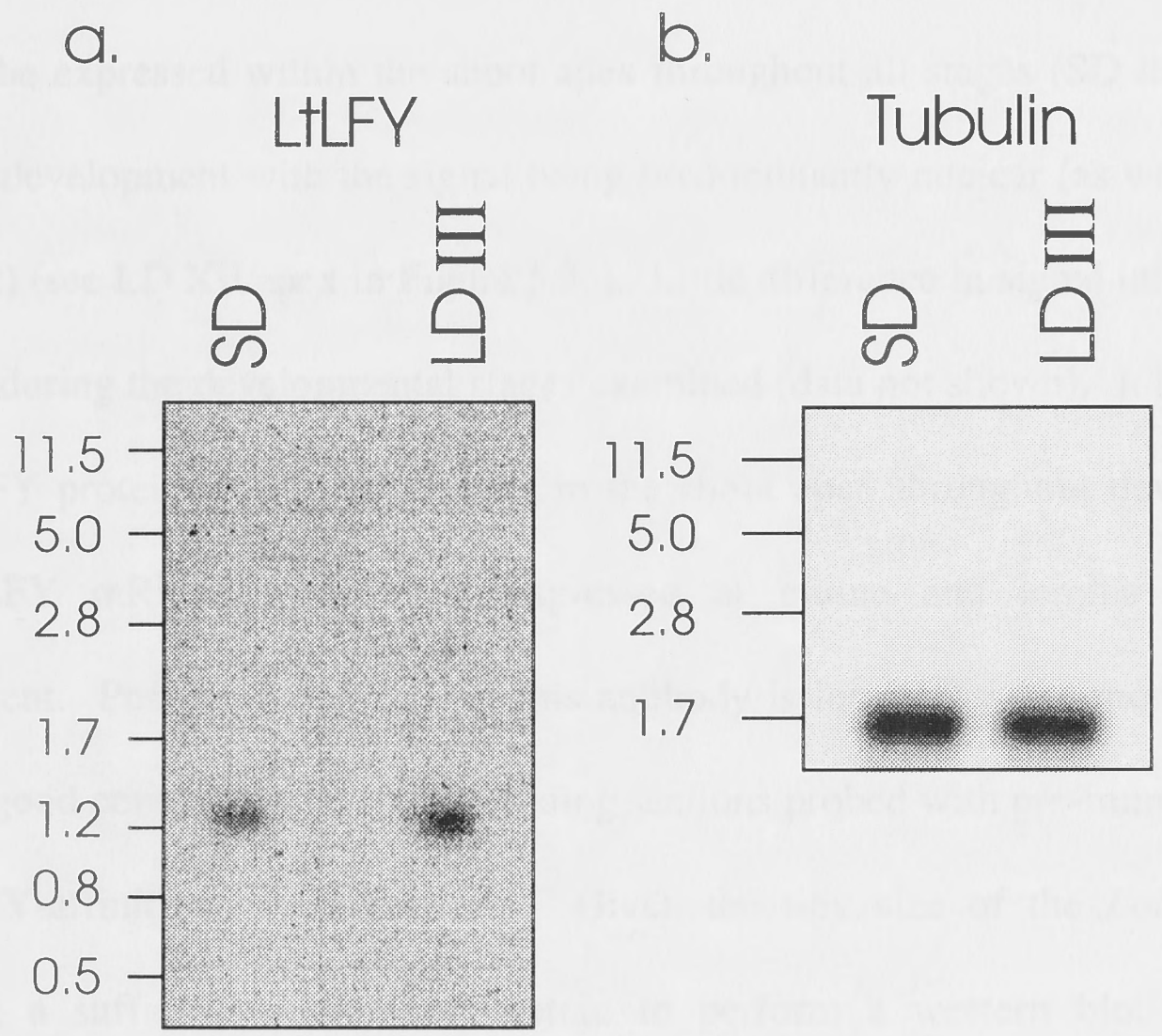


Figure 5.4 Virtual northern blot analysis of vegetative SD and florally induced LD III PCR-based cDNA (24 h after the end of the single LD) from shoot apices hybridised with (a) a fragment of the *LtLFY* gene within exon 3 (LtLFY1/2) or (b) a fragment of a *Lolium* tubulin cDNA (see Table 2.3.4) to compare loading between lanes. Sizes in kbp are indicated to the left.

ranging in age from SD vegetative through LD XXX. This antibody was raised against an *E. coli*-produced LEAFY protein from *Arabidopsis*. Immunolocalisation was performed as detailed in Appendix II.

Using this serum (without affinity purification), LEAFY protein was found to be expressed within the shoot apex throughout all stages (SD through LD XXX) of development with the signal being predominantly nuclear (as was the case for CDC2) (see LD XII apex in Figure 5.3c). Little difference in signal intensity was observed during the developmental stages examined (data not shown). It is puzzling why LtLFY protein would be detected in the shoot apex throughout development, with LtLFY mRNA only highly expressed at glume and lemma stages of development. Possibly specificity of this antibody is lost across the monocot/dicot split and good controls are needed including sections probed with pre-immune serum and LtLFY-affinity purified antibody. Given the tiny size of the *Lolium* apex, extracting a sufficient amount of protein to perform a western blot would be unrealistic. If the antibody is LEAFY-specific, then LtLFY protein must be imported to the shoot apex from elsewhere in the plant. On the other hand it is likely that wheat was a major dietary component for the rabbits used to generate the antibodies. Hence detection of non-leafy *Lolium* proteins highly related to wheat proteins is a possibility. With these issues unresolved, the LEAFY immunolocalisation data is less credible.

Discussion

5.4 *Comparison of LtLFY with Homologues from Other Species*

5.4.1 *Nucleotide and Amino Acid Comparisons*

Based on the DNA gel blot analysis, *LEAFY* is a single-copy gene in *Lolium* (see Figure 5.2). This gene is also single copy in the diploid genomes of

Arabidopsis and *Antirrhinum* (Coen *et al.*, 1990; Weigel *et al.*, 1992), but in *Nicotiana* diploid and tetraploid lines, two and four copies were identified respectively (Kelly *et al.*, 1995). Extensive sequence conservation at both the nucleotide and amino acid levels is observed when the partial LtLFY sequence (within exon 3) is compared with that for other LEAFY homologues (see Figures 5.1b and 5.5). Not surprisingly, over a region of 25 amino acids, LtLFY and ZmLFY are more similar to each other than to other LEAFY homologues with only 3 conservative changes observed between them (Weigel and Meyerowitz, 1993 and see Figure 5.5 and Figure 5.6). In a PILEUP analysis (GCG [Genetics Computer Group] program, Version 8, Madison, Wisconsin, USA) of several LEAFY amino acid sequences, as might be expected, the monocot sequences are more closely related to the dicot sequences than to those of non-angiosperm species (see Figure 5.6).

5.4.2 *Expression of LtLFY Compared to that for Other LEAFY Homologues.*

Another level by which homology can be assessed is with respect to pattern of expression. *In situ* analysis of LEAFY mRNA expression had not previously been determined for a monocot. In this study, detectable expression of LtLFY was more transient and significantly different than expression of its homologues in *Arabidopsis*, *Antirrhinum*, tobacco or cauliflower. For *Lolium*, LtLFY mRNA was only detected at the glume and lemma stages (see Figure 5.3). At these stages, expression was localised within the lateral and terminal spikelet meristems, which are inflorescence meristem equivalents. Expression at this stage is just prior to terminal differentiation of the spikelet which occurs when floret sites differentiate alternately along the rachilla. This pattern of expression within the inflorescence meristems at a stage just preceding floral (floret) meristem initiation is

Table 5.1 Summary of the expression of LEAFY homologues from several species determined by *in situ* hybridisation.

		Transcripts detected in:							
					Br	**Se**	**Pe**		
Species	**Clone**	**VM**	**IM**	**FM**	**Gl**	**Le\Pa**	**Lo**	**St**	**Ca**
Lolium temulentum	LtLFY	-	+	-	+	+	-	-	-
Antirrhinum majus	FLORICAULA	-	+	+	+	+	+	-	+
Arabidopsis thaliana	LEAFY	-	-	+	N.P.	+	+	+	+
Nicotiana	NFL1(2)	+	+	+	+	+	+	-	-
Brassica oleracea	BoFH	-	+	+	+	-\-	-	-	-

VM = vegetative meristem; IM = inflorescence meristem; FM = floral meristem; Br = bract; Gl = glume; Se = sepal; Le = lemma; Pa = palea; Pe = petal; Lo = lodicule; St = stamen; Ca = carpel; N.P. = not present. Table compiled from the results of Coen *et al.* (1990; *Antirrhinum majus*), Weigel *et al.* (1992; *Arabidopsis thaliana*), Kelly *et al.* (1995; *Nicotiana*) and Anthony *et al.* (1996; *Brassica oleracea*).

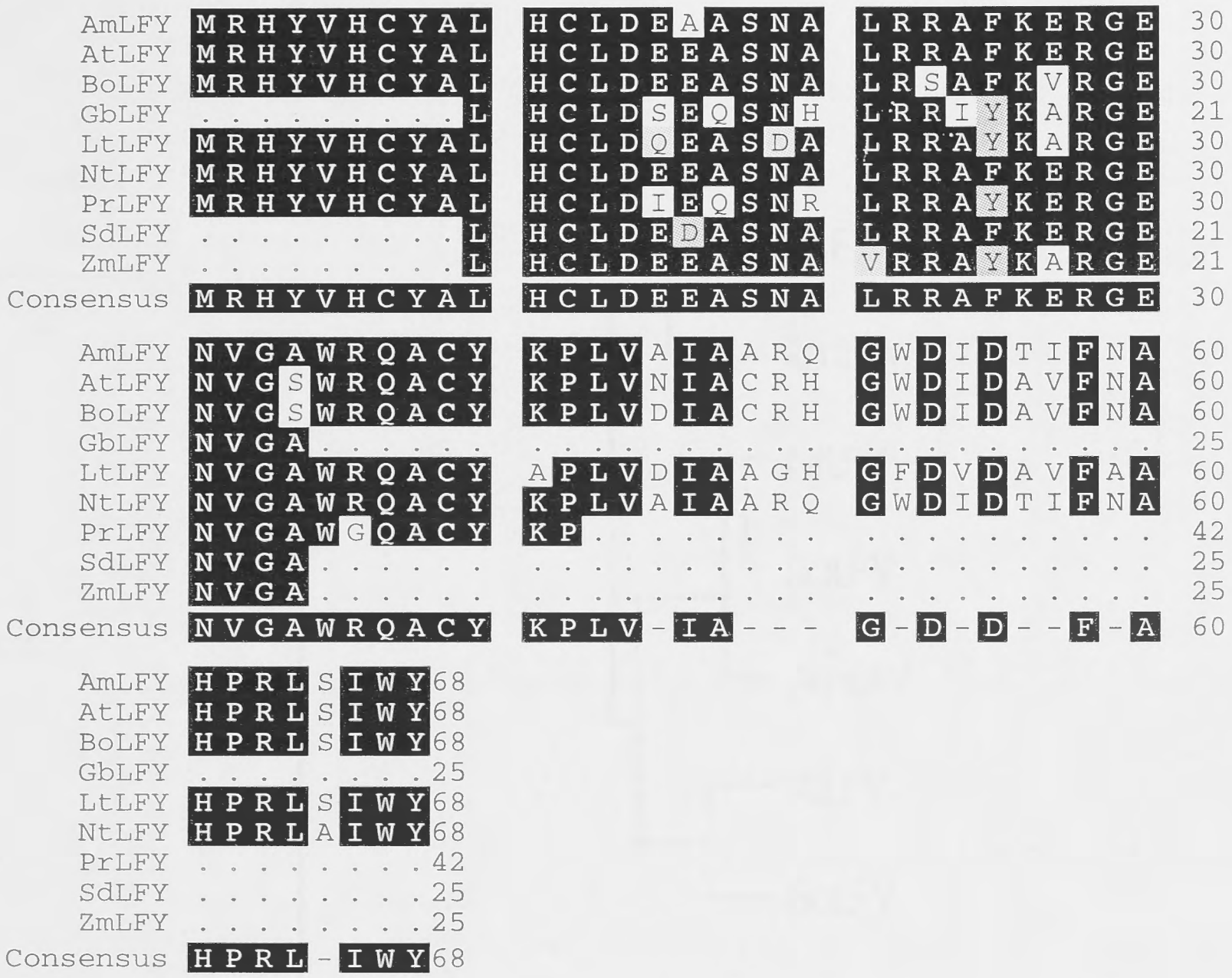


Figure 5.5 Alignment of the predicted amino acid sequence of LtLFY1/2 within a region of exon three for LEAFY homologues from several plant species. Predicted amino acid sequences for Gb, Sd, Zm were from Weigel and Meyerowitz (1993) and the accession numbers for Am, At, Bo and Nt are shown in Figure 5.1b. Dots indicate gaps to maximise alignment. Amino acids that are identical in five or more of the proteins are blocked while those which are similar are shaded. For simplicity, *FLORICAULA* has been renamed to AmLFY and *NFL* renamed to NtLFY. Am is *Antirrhinum majus*; At is *Arabidopsis thaliana*; Bo is *Brassica oleracea*; Gb is *Ginkgo biloba*; Lt is *Lolium temulentum*; Nt is *Nicotiana tabacum*; Pr is *Pinus radiata*; Sd is *Solanum dulcamara*; and Zm is *Zea mays*.

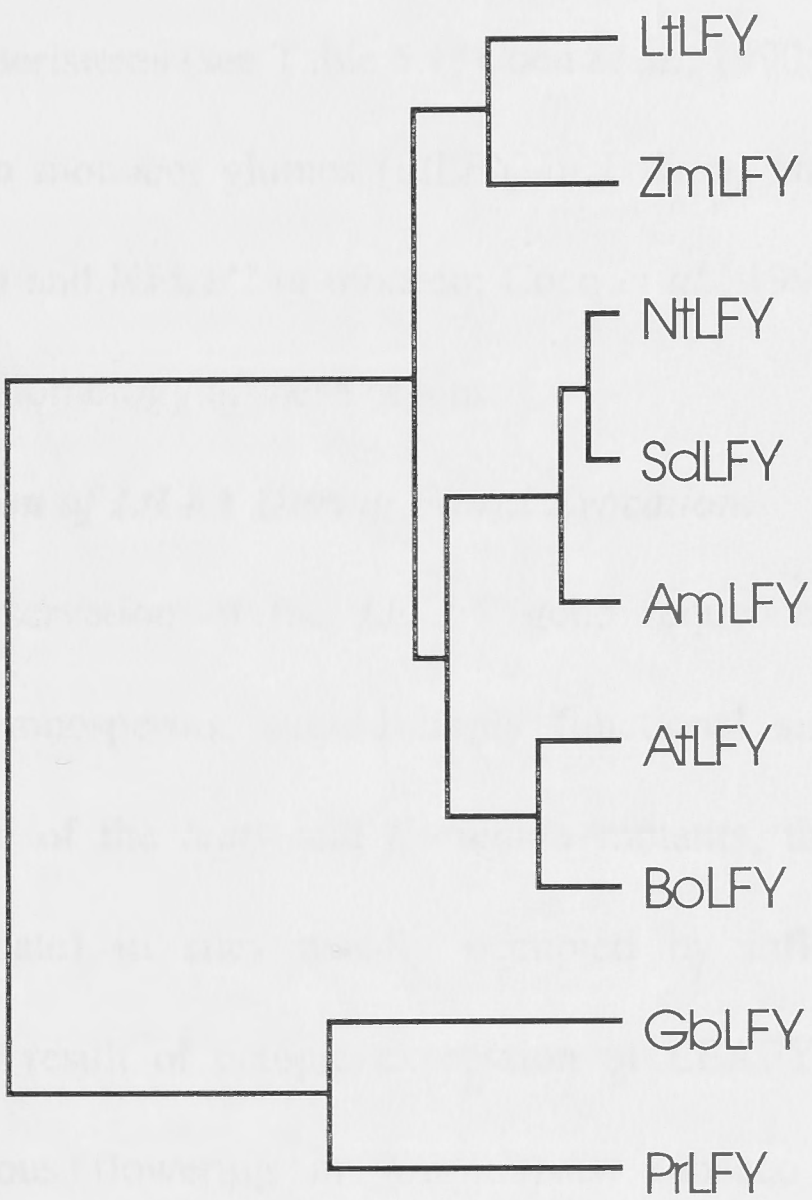


Figure 5.6 Tree showing the evolutionary relationship of predicted amino acid sequences for plant LEAFY homologues within a fragment of exon 3.

The tree was constructed using the PILEUP analysis of the GCG program. Predicted amino acid sequences for Gb, Sd, Zm were from Weigel and Meyerowitz (1993) and the accession numbers for Am, At, Bo and Nt are shown in Figure 5.1b. For simplicity, *FLORICAULA* has been renamed to AmLFY and *NFL* renamed to NtLFY. Am is *Antirrhinum majus*; At is *Arabidopsis thaliana*; Bo is *Brassica oleracea*; Gb is *Ginkgo biloba*; Lt is *Lolium temulentum*; Nt is *Nicotiana tabacum*; Pr is *Pinus radiata*; Sd is *Solanum dulcamara*; and Zm is *Zea mays*.

most similar to the expression of the LEAFY homologue, *BoFH*, in cauliflower (see Table 5.1; Anthony *et al.*, 1996). The absence of LtLFY expression within the floret meristems certainly contrasts the expression of FLO and LEAFY which are only detectable in floral meristems (see Table 5.1; Coen *et al.*, 1990; Weigel *et al.*, 1992). LEAFY expresses in monocot glumes (LtLFY in *Lolium*) and in bracts of dicots (FLO in *Antirrhinum* and NFL1/2 in tobacco; Coen *et al.*, 1990; Kelly *et al.*, 1995) which could suggest homology of these organs.

5.4.3 *Expression of LtLFY During Floral Evocation.*

The conservation of the *LEAFY* gene sequence between monocots, dicots and even gymnosperms, should imply functional similarity. The more vegetative phenotype of the *leafy* and *floricaula* mutants, the initiation of floral meristems (determinate) in sites usually occupied by inflorescence meristems (indeterminate) as a result of ectopic expression of LEAFY in *Arabidopsis* and aspen, and precocious flowering in *Arabidopsis*, tobacco and aspen LEAFY overexpressors, as well as the endogenous expression of LEAFY in floral tissue all indicate that this gene has a role during reproductive development (Coen *et al.*, 1990; Weigel *et al.*, 1992; Weigel and Nilsson, 1995).

To date daylength-induced changes in the expression of LEAFY homologues using *in situ* hybridisation have been examined only for the short day plant (SDP) *Nicotiana tabacum* cv. Maryland Mammoth (Kelly *et al.*, 1995) as well as in the LDP's *Antirrhinum* (Bradley *et al.*, 1996) and *Lolium*. For *Arabidopsis* no information has been published on induction of LEAFY by daylength. In *Lolium*, there is a marked increase of expression of LtLFY 9 to 12 days after single LD induction, whereas in *Antirrhinum*, FLO expression was detected within 1 to 2 days after transfer to LD photoperiods (see Figure 5.3; Bradley *et al.*, 1996).

Within vegetative shoot apices of *Antirrhinum* the expression of FLO is not detectable, whereas LtLFY in *Lolium* and LEAFY in *Arabidopsis* are expressed to a low level (Coen *et al.*, 1990; Figure 5.4; Yanofsky, unpublished lecture, Lorne, Australia). By contrast, expression of LEAFY homologues to reasonably high levels is evident in the vegetative meristem of a SD variety of tobacco, in *Sinapis* shoot tissue prior to LD induction, and in *Populus* prior to flowering (Kelly *et al.*, 1995; Melzer *et al.*, 1995; Strauss *et al.*, 1995). These observations seem to argue against a role for LEAFY in photoperiodically regulated floral induction. However a number of lines of evidence challenge this conclusion: (i) studies with mutants of *Arabidopsis* and snapdragon show that LEAFY is essential (Coen *et al.*, 1990; Weigel *et al.*, 1992), (ii) LEAFY is expressed only to a low level in *Arabidopsis* and *Lolium* vegetative apices, but increases in LD, and (iii) in tobacco and aspen (a hybrid of *Populus tremula* and *Populus tremuloides*), species in which LEAFY is expressed vegetatively, flowering occurs earlier with overexpression of the *LEAFY* gene (Weigel and Nilsson, 1995). Translation of LEAFY message may be inefficient in species which express LEAFY under non-inductive conditions or at times well before flowers initiate. Alternatively, LEAFY action may be suppressed under these conditions. Finally, interactions between *LEAFY* and other flowering genes may be necessary for LEAFY action. This is supported by the fact that LEAFY overexpressors in *Arabidopsis* do not flower as early as the *emf1* single or *elf3*/hy2 double mutants (Sung *et al.*, 1992; Zagotta *et al.*, 1996).

It may also be that LD up-regulates LEAFY expression in species regardless of whether they are typically short or long day plants. Long day-induced expression of LEAFY homologues in floral shoot apices of *Lolium*, *Arabidopsis* and *Antirrhinum* as well as expression of NFL message in vegetative shoot apex of the

SD tobacco cv. Maryland Mammoth (which are grown under non-inductive LD photoperiods) supports this suggestion. Furthermore, as long photoperiods increase GA level then, as discussed later, increased LEAFY expression may be GA-mediated. Insufficient data exists to draw such conclusions for *Sinapis alba* and aspen.

Conclusions

A conserved fragment of the *LEAFY* gene was isolated from *Lolium*, present as a single copy gene exhibiting striking nucleotide and amino acid identity with all known homologues of LEAFY. Despite this, its expression within the spikelet meristems and glume primordia does not match the pattern of expression for other cognate LEAFY homologues and might indicate that its function is unique. Although LtLFY is up-regulated by LD floral induction, this increased expression is not detected until well after the "induction event." Later in development LtLFY is expressed within provascular tissue in the centre of the shoot apex as well as in the spikelet sites.

CHAPTER 6

ROLE OF GAMYB IN FLORAL EVOCATION OF *LOLIUM*

Introduction

MYB proteins were first identified in animals as regulators of proliferation and differentiation in blood cell lines (see Martin and Paz-Ares, 1997). Although animal MYB's have three imperfect repeats (R1, R2 and R3) of ca. 50 amino acids each, their counterparts in plants usually only have two (R2 and R3) (Martin and Paz-Ares, 1997). These repeats determine the DNA binding specificity of each protein (Martin and Paz-Ares, 1997). Several plant MYB's are transcriptional activators within monocot and dicot flowers with many controlling the transcription of enzymes required for phlobaphene and anthocyanin biosynthesis (flavonoid pigments) (Jackson *et al.*, 1991; Avila *et al.*, 1993; Grotewold *et al.*, 1994). Three MYB's, *PhMYB3* from petunia and the product of the *MIXTA* gene of *Antirrhinum* and its orthologue *PhMYB1* from petunia are expressed specifically in petals, with the former likely controlling anthocyanin biosynthesis and the latter two affecting epidermal cell shape (Noda *et al.*, 1994; Solano *et al.*, 1995).

The gibberellin (GA) class of plant growth regulators have long been considered to play a role in flowering (Lang, 1965). Of all the substances which have been applied to a wide variety of plant species under strictly non-inductive or marginally-inductive conditions, the GA's have been generally shown to effectively promote floral induction, initiation and/or floral developmental processes (reviewed in Pharis and King, 1985). Many long day plants (LDP's) respond to the GA class of molecules by flowering under non-inductive short days including *Agrostemma*, spinach, *Thlaspi* and *Lolium*. Also the levels of endogenous GA's and bioactive

GA-like substances are elevated after exposure to long photoperiods compared with their levels under continuous SD's (Zeevaart, 1971; Jones and Zeevaart, 1980a; Jones and Zeevaart, 1980b; Metzger and Zeevaart, 1982; Metzger, 1985; Pharis *et al.*, 1987; Talon and Zeevaart, 1990; Zeevaart and Gage, 1993). Associated with the vegetative to floral transition in LDP's, the stem may elongate (bolt) substantially, but this is not always true, and does not occur in *Lolium temulentum* until three weeks after the LD.

Increased GA status may thus represent one component/cue which elicits the vegetative to floral transition in LDP's such as *Lolium*. Furthermore, the ability of applied GA's to induce flowering is unrelated to their ability to increase internode elongation (Evans *et al.*, 1990). For *Lolium*, the GA's most effective at inducing flowering under totally non-inductive SD conditions are often relatively ineffective at causing internode elongation (Evans *et al.*, 1990; Evans *et al.*, 1994).

Molecular genetic evidence has provided additional support for a role of GA in LD-induced flowering. Mutants of the LDP *Arabidopsis* with very reduced GA levels (e.g., *ga1*-3) are extremely delayed in their flowering under short days (Wilson *et al.*, 1992). Thus GA is essential for its flowering under SD's. In contrast, the *spindly* (*spy*) and *elongated* mutants of *Arabidopsis*, which have the appearance of being treated with a high level of GA, flower early (Jacobsen and Olszewski, 1993; Halliday *et al.*, 1996; Jacobsen *et al.*, 1996). In barley, the *slender* mutant also flowers early and it too has a phenotype which is consistent with the constitutive GA response of the *spindly* mutant (Chandler, personal communication).

How GA's regulate flowering is still unclear, but genes controlling flowering of *Arabidopsis* are also responsive to GA. For example, the promoter of the transcription factor LEAFY is GA responsive (Blázquez and Weigel, personal

communication). Further, heterozygous *lfy*-6 mutants (*lfy*-6 being a null allele) show weak or incomplete vegetative to floral transitions which are partially reversed in *lfy*-6(+/-) *spy*-2 or *spy*-3 double mutants, or by application of GA_3 (Okamuro *et al.*, 1996). Therefore, it might be that GA acting through LEAFY induces flowering in *Arabidopsis*.

A further critical link between GA and gene floral expression may involve the transcription factor GAMYB which has been shown to be a component of GA-mediated signal transduction in cereal aleurone (Gubler *et al.*, 1995). They found that GAMYB is a transcriptional activator of an *α-amylase* gene in barley aleurone cells through binding to a GA-response element (TAACAAA) with its promoters. As yet, there is no evidence to indicate whether GAMYB is a component of the GA signal transduction pathway in other cell types. This chapter aims to test whether GAMYB is expressed in *Lolium* apices and if so, whether it is up-regulated during the vegetative to floral transition, thereby implying it may have a role in another GA-response.

Results

6.1 *A GAMYB-like Gene is Up-regulated in Florally-Induced Shoot Apices of Lolium temulentum.*

To determine if *GAMYB* or other MYB-like transcription factors are expressed in shoot apex tissue following LD floral induction, two types of probes were hybridised at intermediate stringency (0.5 X SSPE; 0.2% SDS at 65°C) to virtual, PCR-based, northern blots of cDNA from vegetative (SD) and florally evoked (LD III) apices. The first probe was derived from the 5'-end of barley HvGAMYB (nucleotide 272 to 1121) and contained the region encoding the DNA-

binding R2/R3 repeats which are conserved in all plant MYB-like transcription factors. The second probe was derived from the 3'-end of the HvGAMYB cDNA (nucleotide 1198 to 2189) and had previously been shown to be gene specific (Gubler *et al.*, 1995). For each probe, several hybridising bands were observed in the SD and LD III lanes with the hybridisation strength increasing following floral induction (see Figure 6.1). Therefore, the expression of MYB-like genes is increased during LD floral evocation. The largest hybridising band had a length of about 2.8 kbp which is approximately the expected size for transcripts of the *GAMYB* gene.

6.2 *Cloning of the Lolium GAMYB Homologue*

Putative florally up-regulated MYB transcripts were identified from duplicate lifts of a PCR-based cDNA library, constructed from *Lolium* LD III shoot apices, by hybridising the 5'-R2/R3-containing fragment at the same stringency used to probe the virtual RNA blots. (See section 2.2 for the detailed method for PCR-based library construction and screening.) Two plaques in 360 000 specifically hybridised with the barley GAMYB probe.

Both plaques were excised and sequenced. The sequence of these clones was identical in regions where they overlapped. Interestingly, based on sequence of the *HvGAMYB* gene (Kalla and Gubler, unpublished data), one clone was truncated within intron 1. Neither clone was full length at its 5'-end. (The author greatly appreciates technical assistance of Robyn Watts and Cheryl Blundell in the isolation and sequencing of the *Lolium* GAMYB clones.)

The 5' end of the *Lolium* MYB-like gene was obtained by PCR from dT-primed cDNA synthesised from 25 LD VI (double ridge stage) shoot apices (see sections 2.2.1 and 2.2.2). The primer pair 001 Hind [5'-d(ATAAGCTTGAGATGT-

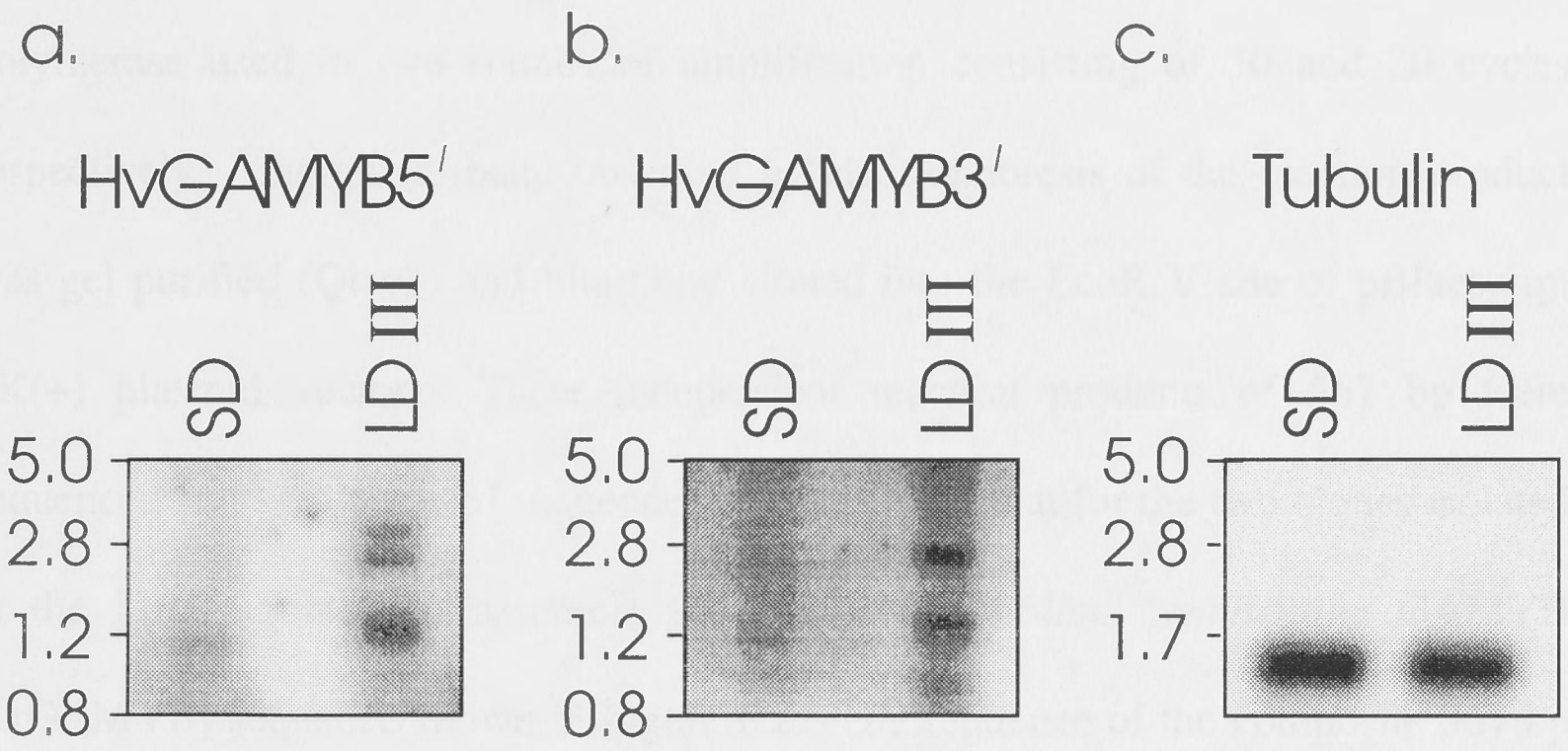


Figure 6.1 Virtual northern blot analysis of vegetative SD and florally induced LD III PCR-based cDNA (24 h after the end of the single LD) from shoot apices hybridised with (a) a fragment towards the 5' end of the HvGAMYB cDNA (nucleotide 272 to 1121) within the DNA-binding R2/R3 repeats, (b) a gene specific fragment from the 3'-end of HvGAMYB (nucleotide 1198 to 2189) or (c) a fragment of a *Lolium* tubulin cDNA (see Table 2.3.4) to compare loading between lanes. Sizes in kbp are indicated to the left.

ACCGGGTGAAGAGCGAGAGC)-3'], designed based on the sequence of barley GAMYB near the start codon (Gubler, unpublished), and Lo8 [5'-d(AAAGACCAT-TCCCATTCAGA)-3'], complementary to sequence of the *Lolium* MYB-like gene outside the R2/R3 repeat, was used (primer locations shown in Figure 6.2). Reaction conditions for PCR amplification were as detailed in sections 2.3.7, with *Pfu* DNA polymerase used in two rounds of amplification consisting of 30 and 20 cycles respectively. The single band observed by electrophoresis of the reaction product was gel purified (Qiaex) and blunt end cloned into the EcoR V site of pBluescript SK(+) plasmid vector. Three independent reaction products of 567 bp were sequenced and the corrected sequence combined with that for the two clones isolated in the library screen to assemble the full length *Lolium temulentum* GAMYB (LtGAMYB) sequence shown in Figure 6.2. The sequence of the composite 567 bp product was identical over 114 bp with that at or near the 5' end of each cDNA clone isolated in the library screen. The nucleotide sequence encoding the first 9 amino acids of the LtGAMYB sequence is uncertain since it is identical to that of the primer, although in theory the 3' to 5' exonuclease activity of *Pfu* DNA polymerase should correct mismatches between the primer and the LtGAMYB cDNA. Within the region covered by the 001 Hind primer, the rice and barley GAMYB sequences only differ by a single nucleotide.

6.2 *Support for Homology of GAMYB Genes in Lolium and Barley*

Over the open reading frame, the nucleotide sequence of the *Lolium* GAMYB clone was 91% identical to either the barley or rice GAMYB sequences, with their proteins being 95% and 94% similar, respectively. Within the DNA-binding R2/R3 repeat region (underlined in Figure 6.2), the amino acid sequence of

```
                001 Hind
                _______
1    AGATGTACCGGGTGAAGAGCGAGAGCACCTGCGAGATGATGCACCAGGACCAGATGGATT   60
1      M  Y  R  V  K  S  E  S  T  C  E  M  M  H  Q  D  Q  M  D  S   20

61   CGCCGCTGGCCGACGACGGCGGCGGCAGCAGCGGGGGGTCGCCTCACAGGGGCGGCGGGC  120
21     P  L  A  D  D  G  G  G  S  S  G  G  S  P  H  R  G  G  G  P   40

121  CCCCGCTCAAGAAAGGCCCCTGGACGTCGGCGGAGGACGCCATCCTGGTGGACTACGTCA  180
41     P  L  K  K  G  P  W  T  S  A  E  D  A  I  L  V  D  Y  V  K   60

181  AGAAGCACGGCGAGGGGAACTGGAACGCGGTGCAGAAGAACACCGGGCTCTTCCGCTGCG  240
61     K  H  G  E  G  N  W  N  A  V  Q  K  N  T  G  L  F  R  C  G   80

241  GCAAGAGCTGCCGCCTCCGGTGGGCGAACCACCTCAGGCCCAACCTCAAGAAGGGGGCCT  300
81     K  S  C  R  L  R  W  A  N  H  L  R  P  N  L  K  K  G  A  F  100

301  TCACACCGGAGGAGGAGAGGCTCATCATCCAGCTCCACTCCAAGATGGGCAACAAGTGGG  360
101    T  P  E  E  E  R  L  I  I  Q  L  H  S  K  M  G  N  K  W  A  120

361  CTCGGATGGCTGCTCATTTGCCAGGGCGTACTGATAATGAAATAAAGAATTACTGGAATA  420
121    R  M  A  A  H  L  P  G  R  T  D  N  E  I  K  N  Y  W  N  T  140

421  CTCGAATAAAGAGATGCCAGCGAGCTGGCTTGCCAGTTTATCCTGCTAGTGTAATCAATC  480
141    R  I  K  R  C  Q  R  A  G  L  P  V  Y  P  A  S  V  I  N  Q  160

481  AGTCTGCAAATGAAGATCAGCAGGGCTCCAGTGATTTCAACTGCGGCGAGAATCTTTCTA  540
161    S  A  N  E  D  Q  Q  G  S  S  D  F  N  C  G  E  N  L  S  N  180
                                Lo 8
541  ACAACCATCTGAATGGGAATGGTCTTTATCTACCAGATTTCACCTGTGACAATTTCATTG  600
181    N  H  L  N  G  N  G  L  Y  L  P  D  F  T  C  D  N  F  I  A  200

601  CCAATTCAGAGGCTTTATCTTATGCACCACAGCTCTCAGCTGTTTCAATAAGCAATTTGC  660
201    N  S  E  A  L  S  Y  A  P  Q  L  S  A  V  S  I  S  N  L  L  220

661  TTGGCCAGAGCTTTGCATCAAAACGCTGTGGCTTCATGGATCAAGCTGGGATGCTAAAAC  720
221    G  Q  S  F  A  S  K  R  C  G  F  M  D  Q  A  G  M  L  K  Q  240

721  AGTCTGACAGTTTGCTCCCTGGATTGAGCGATACTGTGAACGGTGCGCTCTCCTCGGTTG  780
241    S  D  S  L  L  P  G  L  S  D  T  V  N  G  A  L  S  S  V  D  260

781  ATCAATTCTCAAACGACTCTGGGAAGCTCAGGCAGGCTCTGGGTTTTGACTACCTCCATG  840
261    Q  F  S  N  D  S  G  K  L  R  Q  A  L  G  F  D  Y  L  H  E  280

841  AAGCCAACTCTAGCAGCAAGATTCTTGCACCCTTTGGCGGTGCGCTTACTGGCAGCCATG  900
281    A  N  S  S  S  K  I  L  A  P  F  G  G  A  L  T  G  S  H  A  300

901  CCTATTTAAATGGCACCTTCTCTGCTTCTAGGTCCACAAATGGTCCTTTGAAGATGGAGC  960
301    Y  L  N  G  T  F  S  A  S  R  S  T  N  G  P  L  K  M  E  L  320
       ▼
961  TCCCTTCACTCCAAGATACCGAATCTGACCCAAATAGCTGGCTCAAGTATACCGTGGCTC 1020
321    P  S  L  Q  D  T  E  S  D  P  N  S  W  L  K  Y  T  V  A  P  340

1021 CTACTATGCACCCTACGGAGTTAGTTGATCCCTACCTTCAGTCTCCAACAGCAACTCCAT 1080
341    T  M  H  P  T  E  L  V  D  P  Y  L  Q  S  P  T  A  T  P  S  360

1081 CGGCGAAATCCGAGTGTGCATCACCAAGGAACAGTGGCCTTTTGGAAGAGCTGCTCCATC 1140
361    A  K  S  E  C  A  S  P  R  N  S  G  L  L  E  E  L  L  H  Q  380

1141 AGGCCGAGGCACTAAGGTCTGGGAAGAACCAGCAGCCGTCTGTGAGAAGCTCAAGCTCCT 1200
381    A  E  A  L  R  S  G  K  N  Q  Q  P  S  V  R  S  S  S  S  S  400

1201 CTGTCAGTACGCCGTGTGATACAACGGTAGTTAGCCCTGACTTTGATCTCTGTCAGGAAT 1260
401    V  S  T  P  C  D  T  T  V  V  S  P  D  F  D  L  C  Q  E  Y  420

1261 ACTGGGAAGAGCATTCCGGTGCTATGCTGCATGAGTATGTTCCTTTTAGCGGTAACTCAC 1320
421    W  E  E  H  S  G  A  M  L  H  E  Y  V  P  F  S  G  N  S  L  440

1321 TGACTGAATCCACCGCTCCTGTGAGCGCGGCATCGCCTGATGCATTTCAGCTCTCCAAAA 1380
441    T  E  S  T  A  P  V  S  A  A  S  P  D  A  F  Q  L  S  K  I  460

1381 TTTCCCCCGAAAGCAATTCGCTGGGATCCGGAGAGCAGGCGATGGAGCCAACGTATGAGC 1440
461    S  P  E  S  N  S  L  G  S  G  E  Q  A  M  E  P  T  Y  E  P  480

1441 CTGGGGCTGGGGATGCTTCACCTCGTCCTGAAAACTTGAGGCCGGACGCGTTCTTCTCCG 1500
481    G  A  G  D  A  S  P  R  P  E  N  L  R  P  D  A  F  F  S  G  500

1501 GGAATACGGCTGATTCGTCCCTCTTCAACAATGCCATAGCAATGCTCCTCGGCAATGACA 1560
501    N  T  A  D  S  S  L  F  N  N  A  I  A  M  L  L  G  N  D  M  520

1561 TGAACACGGAGTGCAAGCCTGGTCTCTTCGATCCTTCCTCCTGGAGCAACATGCCCCATG 1620
521    N  T  E  C  K  P  G  L  F  D  P  S  S  W  S  N  M  P  H  A  540
                                ◜
1621 CTTTCCAAATGTCGGAAGAATTCAAATGAGTTCCTTGCCGAGGCGTGGAAGGAGATTCTT 1680
541    F  Q  M  S  E  E  F  K  *                                   560

1681 GATATCTCTCTGCCGTCCTGATTGTTTTGAGGATAATTTCAGGAAAACTTCATTTGATTG 1740
1741 CAACTCGCTGCTTAGCCGCCACTGACGACGGGTCCTTGACGGCGGATGCAGCTGCTGCCA 1800
1801 GACTTATGTGCTCTTCATGCAACTAGCCCCTTTTCCCCAAATAAAGTTCATGGAGATATA 1860
1861 TGCCGGTT(A)n                                                  1869
```

Figure 6.2 Nucleotide and predicted amino acid sequence of the LtGAMYB cDNA. The EcoR I (◜) and Sac I (▼) restriction sites which are conserved with those in HvGAMYB are indicated with arrowheads and the stop codon is indicated with an asterisk. Primers 001 Hind and Lo8 used to clone the 5' end of LtGAMYB are indicated. The R2 and R3 repeats are shown in bold and are underlined.

Lolium GAMYB compared to that of barley or rice is 99% identical indicating their binding specificities are likely to be very similar. The very high degree of sequence identity of GAMYB clones from barley, *Lolium* and rice suggests that these three genes are homologues (see Figure 6.3).

To provide evidence that the *Lolium* cDNA encoded the barley GAMYB homologue, *Lolium* genomic DNA was cut with three restriction enzymes and analysed by DNA gel blotting using *Lolium* and barley probes synthesised from a conserved EcoR I/Sac I fragment located at the 3'end of each gene (indicated by arrows in Figure 6.2). At high stringency (0.1 X SSPE; 0.2% SDS at 65°C), both the *Lolium* and barley probes hybridised to single bands with similar mobilities for DNA digested with EcoR I or Xho I (See Figure 6.4). For each probe, in the lane containing DNA cut with Bgl II, two bands with similar mobilities were observed resulting from an internal Bgl II site within each probe's sequence. These results indicate that each probe detects the same single copy gene in *Lolium* genomic DNA.

Since HvGAMYB was initially isolated as a transactivator of α-amylase in barley aleurone tissue it was important to determine: (i) whether the MYB expressed in *Lolium* shoot apices was also expressed in *Lolium* de-embryonated half seeds, (ii) whether this mRNA was GA up-regulated, and (iii) whether the increased expression of the *Lolium* MYB-like transcript correlated with an increase in α-amylase message as did GAMYB in barley and rice. Therefore a blot of total RNA from *Lolium* half seeds (a tissue unable to produce endogenous GA) was probed with a ^{32}P-dCTP-labelled LtGAMYB EcoR I/Sac I fragment. These half seeds were incubated in the presence or absence of GA_3 for 6 or 12 h prior to extraction of total RNA. After 12 h α-amylase expression by these half seeds was stimulated by GA_3

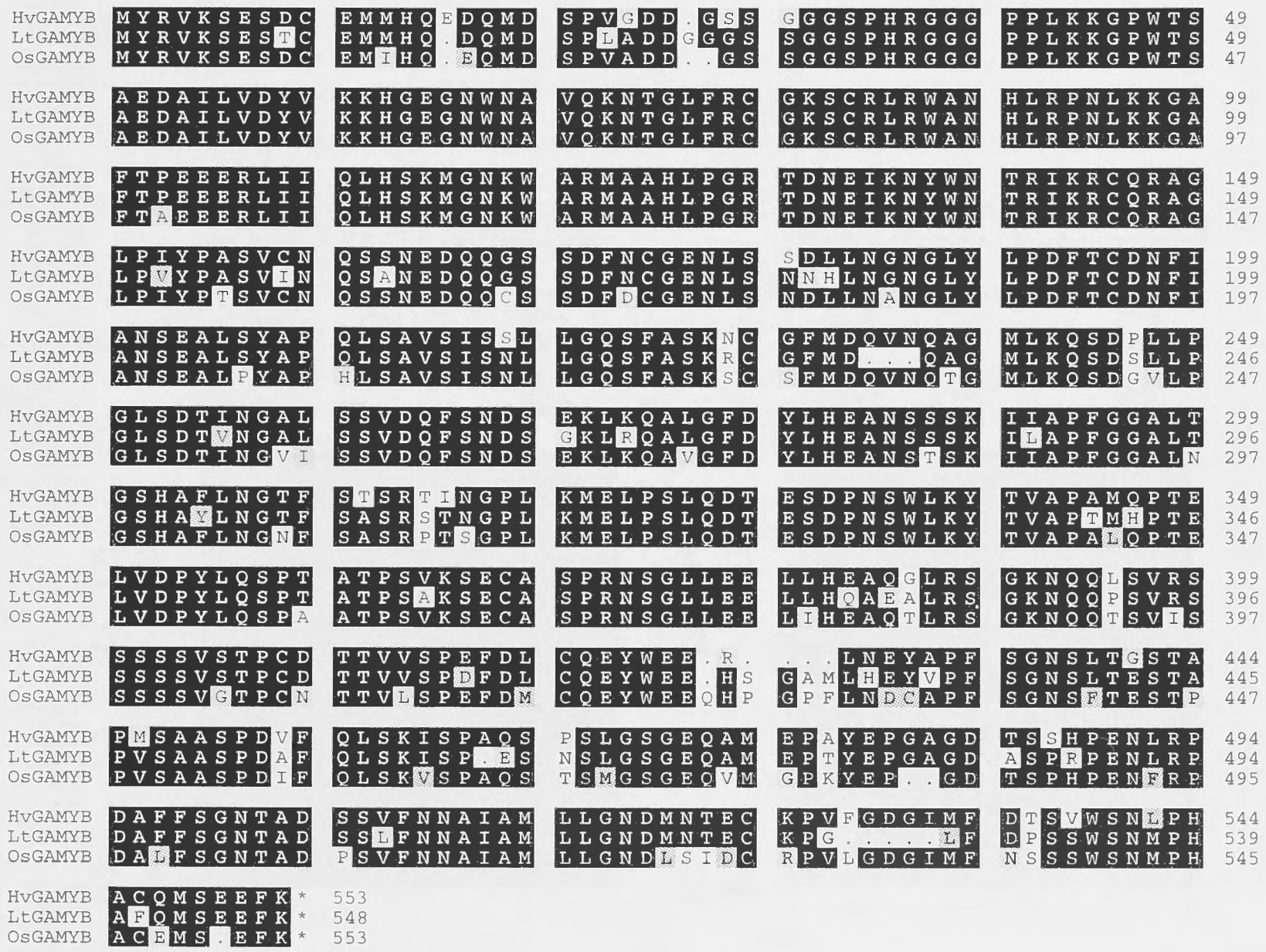


Figure 6.3 Alignment of the predicted amino acid sequences for GAMYB homologues from *Lolium* (Lt), barley (Hv) and rice (Os).

Predicted amino acid sequences for Hv and Os were from Gubler *et al.*, 1995 and 1997 respectively. Dots indicate gaps to maximise alignment. Amino acids that are identical in two or more of the proteins are blocked while those which are similar are shaded.

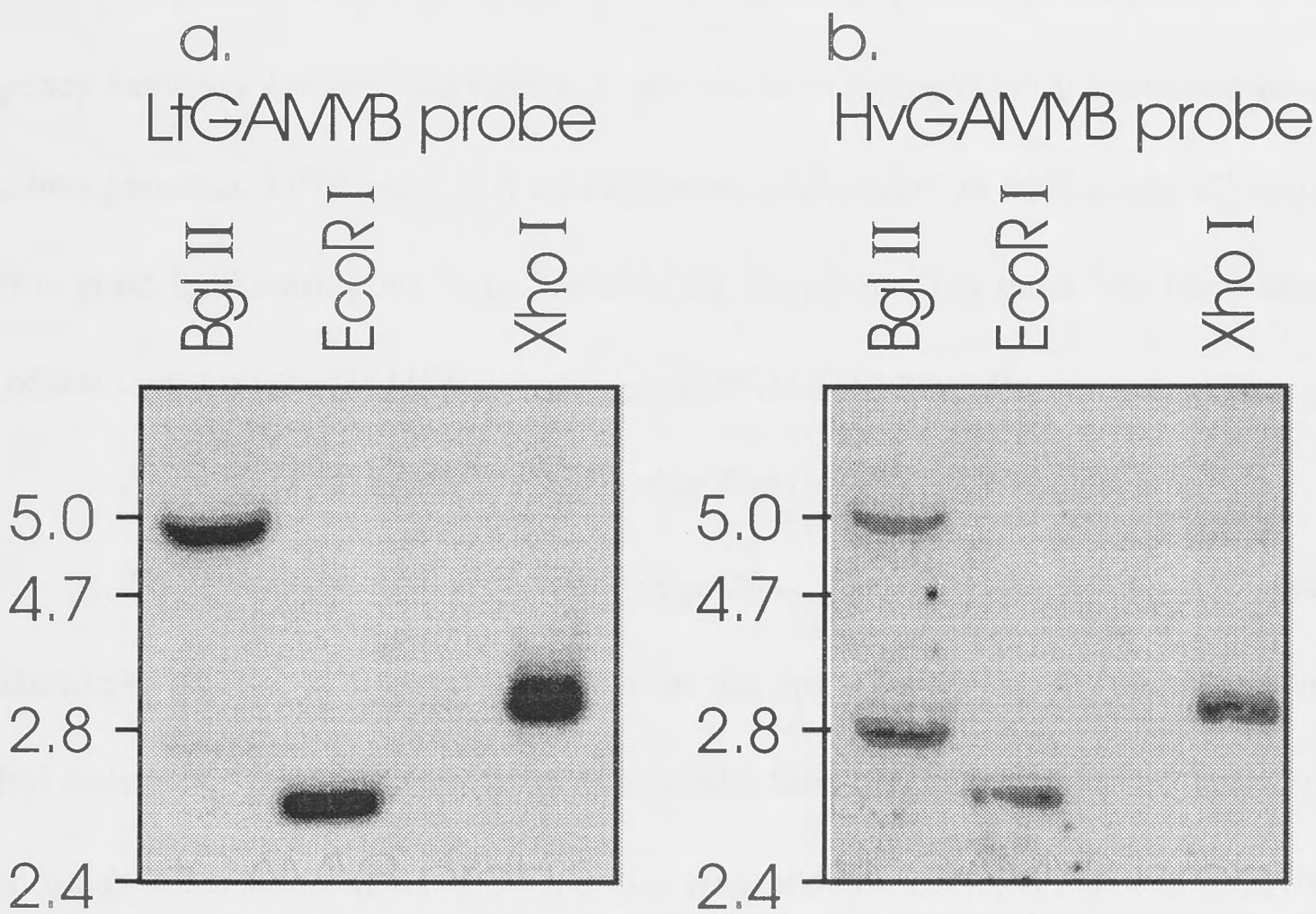


Figure 6.4 *Lolium* genomic DNA gel blot analysis by homologous probes from LtGAMYB and HvGAMYB.

Genomic DNA (20 μg) was digested with Bgl II, EcoR I and Xho I. After blotting, the digested DNA was hybridised with an EcoR I/Sac I restriction fragment from either (a) LtGAMYB or (b) HvGAMYB. This restriction fragment is indicated between the arrowheads on Figure 6.2. Sizes in kbp are indicated to the left.

(data not shown). The *Lolium* MYB-like transcript was expressed in the half seeds (presumably in the aleurone tissue as in barley) and was up-regulated following GA-treatment as has previously been demonstrated for barley and rice (data not shown; Gubler *et al.*, 1995; 1997).

Together, the very high sequence homology, cross hybridisation at high stringency between *Lolium* and barley 3'-probes with a single copy sequence present in *Lolium* genomic DNA, and GA up-regulated expression in half seeds all suggest that this gene is homologous with *HvGAMYB*, therefore this gene has been termed the *Lolium temulentum GAMYB* homologue (*LtGAMYB*) hereafter.

6.3 *Pattern of LtGAMYB Expression During Floral Evocation*

To determine the temporal (vegetative through LD XXX) and spatial localisation of LtGAMYB expression within the apex following floral induction, *in situ* hybridisation was performed on wax-embedded tissue sections as detailed in Appendix I. The EcoR I/Sac I 3'-specific fragment of LtGAMYB was subcloned into the EcoR I/EcoR V sites of pBluescript SK(+) and pBluescript KS(+), in which the multiple cloning site is reversed in the later compared to the former. Since enzymes which leave 3' overhangs, such as Sac I, often generate high background (Ausubel *et al.*, 1988), the Sac I site was made blunt with T4 polymerase prior to digestion with EcoR I and cloning of this fragment. For sense and antisense probe synthesis (see section 2.4), plasmids containing the subcloned fragment were linearised with EcoR I or Hind III respectively and each riboprobe synthesised from the T7 promoter. Colorimetric detection of the hybridising probes was via an anti-DIG Fab fragment conjugated to alkaline phosphatase.

At all timepoints tested and using an equivalent concentration of riboprobe, there was a large and significant difference between signal intensity of the

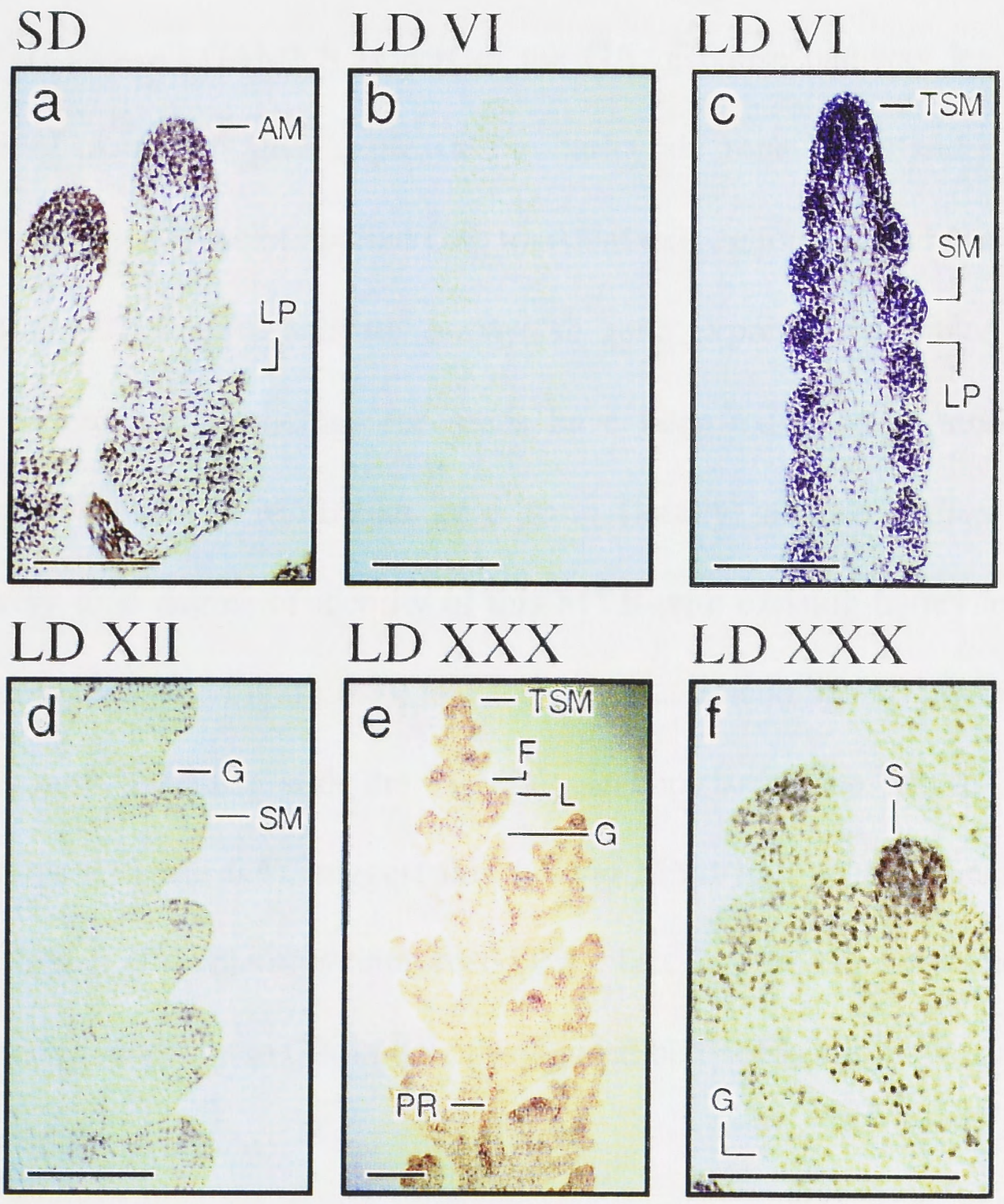
sense control-probed sections and those probed with the antisense probe. A representative section (LD VI apex) hybridised with the sense control riboprobe is shown in Figure 6.5b. There was some hybridisation with the *Lolium* GAMYB sense probe. This background signal was higher than for other sense probes tested by *in situ* hybridisation (compare Figure 6.5b with Figures 3.5c, 4.9a, 4.10c and 5.3a).

Expression of LtGAMYB message was detected throughout the vegetative SD shoot apex, but was highest in the apical dome at the tip of the shoot apex and within the developing leaf primordia at its base (see SD apex in Figure 6.5a). Expression remained unchanged in the shoot apex during the first 12 to 36 h (LD II and LD III) following LD floral induction. Greatest expression for the stages analysed in this study was within the double ridge apex (see LD VI apex in Figure 6.5c). At this stage, expression was highest within the apical dome as well as within the lateral spikelet meristems. In advanced double ridge apices 8 to 11 d (LD IX to LD XII) after induction, expression declined to a lower level but was still detected in the initiating glume primordia as well as within the spikelet sites (see LD XII apex in Figure 6.5d) and in the apical dome (data not shown). At later times (LD XXX), expression remained low and was detected in developing glume primordia as well as the floret meristems and lemma primordia which initiate from these meristems (see LD XXX apex in Figure 6.5e). Of the floral organs which initiate from the floret meristem, highest expression was within the stamen primordia (see LD XXX apex in Figure 6.5f). LtGAMYB was also expressed within the provascular tissue. Especially noticeable are those leading to the developing glumes and lemma at late stages of development (see LD XXX apex in Figure 6.5e).

Figure 6.5 *In* situ localisation of LtGAMYB transcripts in *Lolium* shoot apices.

In situ hybridisations were performed on longitudinal sections of wax embedded apices at different developmental stages using DIG-labelled *in vitro*-transcribed riboprobes synthesised from an EcoR I/Sac I fragment (indicated in Figure 6.2) of the LtGAMYB cDNA. All sectioned apices were from experiment Lt434 (see Figure 2.1). Sections (a), (c), (d), (e), and (f) were hybridised with the antisense riboprobe. A representative section hybridised with the sense control riboprobe is shown in (b). Hybridisations were with an equivalent sense or antisense probe concentration. The same dilution of anti-DIG antibody was used to detect riboprobe hybridisation and the duration of the colour development was identical for all sections in this figure. Hybridisation is visible as the pink to purple product of the alkaline phosphatase reaction. Photographs were taken using Nomarski optics with identical exposures. Therefore, in all antisense hybridised sections, the intensity of staining is directly proportional to the expression of LtGAMYB. Median sections through (a) six-week old vegetative (SD) and (b,c) double ridge stage (LD VI) shoot apices, (d) five spikelet meristems on the flank of the shoot apex at glume stage (LD XII), (e) two spikelet sites at floret stage (LD XXX) and through (f) a stamen primordium on the flank of a floret site (LD XXX). The bars are 100 μm.

Abbreviations: AM, apical meristem; F, floret; G, glume; GP, glume primordium; L, lemma; LP, leaf primordium; PR, provascular strand; SM, spikelet meristem; S, stamen primordium; TSM, terminal spikelet meristem.


SD
LD VI
LD VI
a
AM
LP
b
c
TSM
SM
LP
LD XII
LD XXX
LD XXX
d
G
SM
e
TSM
F
L
G
PR
f
S
G

6.4 *GAMYB is Also Expressed in the Shoot Apex*

Seed germination, flowering and growth are all processes mediated by GA. In germinating monocot seeds, GA synthesised by the embryo activates cells within the aleurone layer to synthesise and secrete hydrolytic enzymes, including α-amylase into the endosperm thereby causing starch digestion. Previously, Gubler *et al.* (1995; 1997) showed GAMYB is part of the GA response pathway leading to transactivation of *α-amylase* gene expression in barley aleurone layers and rice half grains. In fact, in the absence of applied GA, transient expression assays have shown that GAMYB is sufficient to activate *α-amylase* gene expression (Gubler *et al.*, 1995; 1997). Here, these findings for seeds have been extended to studies on flowering by the cloning a MYB-like gene from florally induced *Lolium* shoot apices. The very high degree of identity of this MYB with existing barley and rice GAMYB homologues (see Figure 6.3), and the fact that 3'-end specific barley and *Lolium* probes both hybridise with the same single copy sequence within *Lolium* genomic DNA (see Figure 6.4), suggest this *Lolium* MYB-like gene is the *Lolium GAMYB* homologue. This claim is reinforced by the fact that the *Lolium* homologue is also expressed in response to GA in de-embryonated half seeds with its expression preceding that of α-amylase.

Just as GAMYB has been shown to mediate GA controlled processes during germination, GAMYB might also provide a critical link between GA response and transcriptional activation of genes involved in other GA-mediated processes such as flowering. The expression of GAMYB in seeds and shoot apices appears to be physiologically relevant since both tissues are rich sources of GA (Pharis *et al.*,

1987). Thus the *GAMYB* gene might activate the expression of "flowering" genes in a GA-dependent manner.

There appears to be a correlation between GA levels and *GAMYB* gene expression during flowering. A link between GA's and flowering of *Lolium* has been examined extensively (Evans *et al.*, 1990). Further, although less well characterised, for barley there appears to be a similar link as the *slender* mutant shows GA independence for α-amylase expression, growth (Chandler, 1988) and flowers early (Chandler, personal communication). Although quantitation of endogenous levels of "biologically active" GA's at the shoot apex during monocot inflorescence development has only been assessed by bioassay, in each of barley, *Lolium*, oat, and rice, the level of GA_3-like activity is high at the double ridge stage and falls thereafter (Osada *et al.*, 1973; Nicholls, 1974; Kaufman *et al.*, 1976; Pharis *et al.*, 1987). A second peak of GA_3-like activity is also found during stamen development in barley (Nicholls, 1974), oat (Kaufman *et al.*, 1976) and rice (Osada *et al.*, 1973) (*Lolium* not determined). Also notable is that the florigenic effect of exogenously applied GA_3 is greatest on LD VI (King *et al.*, 1993). Therefore, a correlation exists between high endogenous GA's in monocot inflorescences (spikelets) and the elevated expression of LtGAMYB message (refer back to Figure 6.5). During stamen development GA action may also be important (Sawhney, 1992), and this fits with the high expression of LtGAMYB in young stamens (see Figure 6.5f). Thus, LtGAMYB may regulate the processes of floral evocation and subsequent floral development in response to endogenous GA's of the shoot.

At least over the early stages of floral development in *Lolium*, LtGAMYB may directly activate important floral transcription factors. In apices

from a single timecourse experiment, LtGAMYB expression peaked at the double ridge stage (LD VI; see Figure 6.3), with expression of LtLFY peaking several days later at the advanced double ridge stage (LD IX to LD XII; see Figure 6.3). The presence of a putative GAMYB binding site within the *Arabidopsis LEAFY* promoter (Genbank accession number M91208) suggests that GAMYB may have a role in activating LEAFY (see Figure 6.4). However, it remains to be determined whether this site is functionally important for GA-induced gene expression in *Arabidopsis* and, if so, whether it is mediated through GAMYB. Whether this site is conserved in the promoter of *Lolium LEAFY* also remains to be tested.

MYB consensus binding sequence: YAACKGHH

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Arabidopsis LEAFY promoter site: CAACTGTC

Y = C or T (Pyrimidine)

K = A or T (Keto)

H = A, C or T (not G)

Figure 6.6 Putative MYB-binding site within the *Arabidopsis LEAFY* promoter.

Conclusions

The *Lolium GAMYB* homologue was isolated from a florally induced (LD III) shoot apex PCR-based cDNA library. In addition the *Lolium* sequence has a high homology with the barley and rice *GAMYB* genes and, like the barley and rice homologues, in seeds it is up-regulated by GA with its expression in endosperm tissue preceding that of α-amylase. The *Lolium GAMYB* gene is also expressed in the shoot apex throughout floral development (from SD vegetative to LD XXX floral apices). In vegetative and pre-double ridge shoot apices it is expressed predominantly in the apical dome and within the developing leaf primordia at their base. Highest expression is detected in the double ridge apex in the terminal and lateral spikelet sites. Thereafter, reduced expression is observed at the advanced double ridge stage. Later in development, LtGAMYB is expressed in the glume primordia and floret sites. Of the floral organs, stamen primordia show the highest level of expression. The high level of GAMYB expression in the spikelet meristems and stamen primordia correlate with response to GA during these stages (evocation and stamen development) as well as with the high level of GA found in these tissues of the plant.

CHAPTER 7

OVERVIEW DISCUSSION

Graminaceous plants make up the bulk of economically important crop plants and pasture species. Although the molecular biology of their flowering is relatively unstudied, the LDP *Lolium temulentum* L. strain Ceres provides an inducible system which can be used to examine the vegetative to floral transition in monocots. The benefit of using *Lolium* is not only that it flowers in response to a single LD, but that the physiology of its flowering has been well characterised over the past 40 years.

A framework which can be applied to studying the flowering process in a plant species such as *Lolium* is provided by combining the ABC model of floral development (Weigel and Meyerowitz, 1994), with models for genes controlling time to flower (Weigel, 1995; Amasino, 1996). At the outset of this project it was unknown whether genes important in floral development in dicot species would similarly control this process in monocots. This study, along with those of Schmidt *et al.* (1993) and Mena *et al.* (1995; 1996), have now shown that at least *LEAFY* and the MADS-box class of transcription factors, are important during evocation and floral differentiation in monocots. This is a significant finding since: (i) it provides a beginning by which the timing of the reproductive process in the grasses may be manipulated (examine Weigel and Nilsson, 1995), (ii) it suggests that other classes of genes regulating the vegetative to reproductive transition might also be conserved between monocot and dicot systems, and (iii) it contributes to a pool of data which will allow molecular evolutionary comparison of monocot and dicot floral development.

7.1 *Timing and Localisation of Changes During the Vegetative to Floral Transition*

Previous studies have revealed many physiological, cellular and morphological changes which occur in *Lolium* plants induced to flower with a single long photoperiod. Here, changes in gene expression occurring during the vegetative to floral transition have been characterised. These were combined with the results of previous studies to produce a composite timeline of events from LD induction through floral organ development (see Figure 7.1).

On LD I, florally-responsive seedlings are exposed to a single LD provided as a 24 h-LD by extending an 8 h photosynthetic SD with a 16 h low-irradiance incandescent extension. Midway through this extension, a brief 5-fold increase in the mitotic index occurs within the apical dome (Jacqmard *et al.*, 1993). Near the beginning of LD II, actinomycin D (AD) and 5-flurouracil (5-FU) are most inhibitory to the inductive process (Evans, 1964). Transport of the florigenic signal from the leaves to the apex also begins midway through the extension (Evans and Wardlaw, 1966). On the morning of LD II, increased expression of CDC2aLt is observed which is also when increased rates of ^{32}P and ^{35}S incorporation are observed within the shoot apex (Knox and Evans, 1968; Evans and Rijven, 1967). CDC2aLt shows the first significant change becoming expressed in more cell layers within the apical dome and subsequently in the epidermal and subepidermal layers along the length of the shoot apex. By extrapolation, the rate of primordium initiation from the apical dome begins to accelerate on LD II (Evans and Blundell, 1996). Although. this acceleration is not floral specific, in florally responsive seedlings the number of spikelet sites doubles from 12 to 23 over the next few days. Assessed by culture *in vitro*, on LD III (48 h after the end of the LD) shoot apices

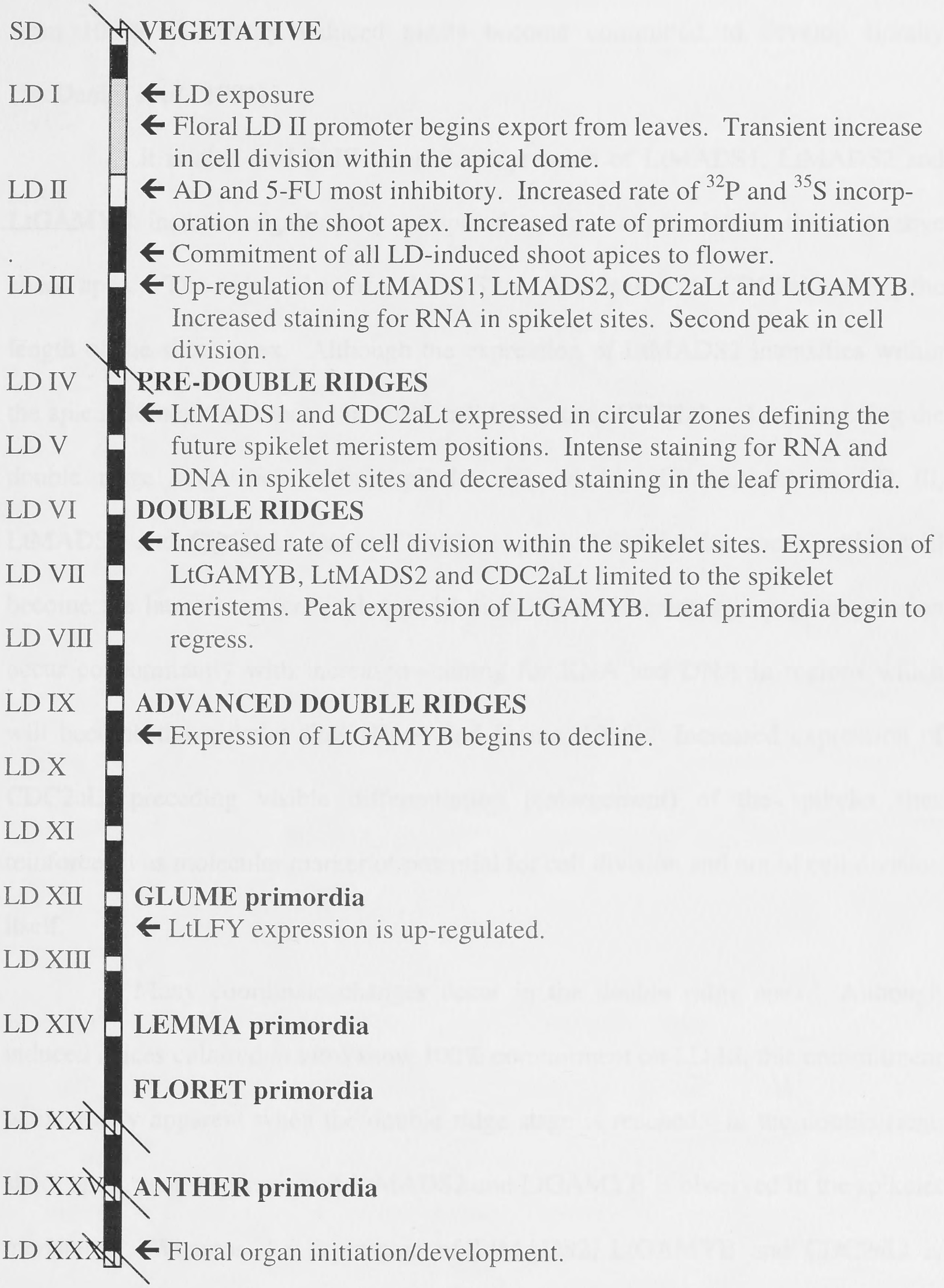

Figure 7.1 Timeline of events occurring in *Lolium temulentum* L. strain Ceres seedlings, grown under high irradiance, during the vegetative to floral transition following single LD floral induction. The inductive 16 h non-photosynthetic extension of the 8 h SD is indicated with shading with days shown in white and nights in black. Events are positioned where they were detected. Stages of floral development are shown in bold capital letters. AD is actinomycin D; 5-FU is 5-fluorouracil.

from 100% of florally induced plants become committed to develop florally (McDaniel *et al.*, 1991).

It is also on LD III when the expression of LtMADS1, LtMADS2 and LtGAMYB increase significantly compared to their expression in the vegetative shoot apex. The expression of LtMADS2 co-localises with CDC2aLt along the length of the shoot apex. Although the expression of LtMADS2 intensifies within the apical dome, it is detected in fewer cell layers than CDC2aLt. Just preceding the double ridge stage (ie., before spikelet sites visibly differentiate) on LD III, LtMADS1 and CDC2aLt become highly expressed in circular zones which will become the lateral and terminal spikelet sites. These increases in gene expression occur concomitantly with increased staining for RNA and DNA in regions which will become the spikelet sites (Knox and Evans, 1966). Increased expression of CDC2aLt preceding visible differentiation (enlargement) of the spikelet sites reinforces it as molecular marker of potential for cell division and not of cell division itself.

Many coordinate changes occur in the double ridge apex. Although induced apices cultured *in vitro* show 100% commitment on LD III, this commitment is externally apparent when the double ridge stage is reached. In the double ridge shoot apex, peak expression of LtMADS2 and LtGAMYB is observed in the spikelet meristems. Whereas the expression of LtMADS2, LtGAMYB and CDC2aLt is restricted to the spikelet meristems at an early stage, LtMADS1 expression does not disappear from the regressing leaf primordia until the advanced double ridge stage. The expression of the former three genes corresponds with the heavy staining of RNA and DNA within the spikelet sites and reduced staining in the leaf primordia in

double ridge apices (Knox and Evans, 1966). In the days which follow, the spikelet meristems continue to expand and the leaf primordia continue to regress.

At the advanced double ridge stage, the expression of LtMADS1, LtMADS2, LtGAMYB and CDC2aLt is reduced from encompassing the entire spikelet meristem to being expressed in the growing tip, the dorsal side of the spikelet meristem nearest the rachis. This is the first stage when expression of LtLFY is detectable by *in situ* hybridisation. LtLFY expression is observed in the dome of the spikelet meristem and in the lemma primordia (when they initiate) and in the tips of the glume primordia. LtMADS1, LtMADS2, CDC2aLt and LtGAMYB were also localised within the lemma primordia and tips of the glumes at this stage. The late (ca. LD XII) up-regulated expression of LtLFY presents a striking contrast to the early (ca. LD III) up-regulated expression of LtMADS1, LtMADS2, LtGAMYB and CDC2aLt. This shows that in *Lolium* unlike *Arabidopsis*, there is a significant time lag between floral induction and the up-regulated expression of LEAFY.

Following the initiation of lemma primordia, at the stage when floret meristems begin to differentiate along the spikelet axis (rachilla), expression of LtLFY becomes undetectable. Later in development, all of LtMADS1, LtMADS2, CDC2aLt and LtGAMYB are expressed in lemma and floret primordia at all stages assessed in this study. Whilst LtMADS1 and LtMADS2 transcripts were both present uniformly in the organs which differentiate from the floral meristem, at late stages of floral development, LtMADS2 eventually became excluded from the reproductive organs and continued to be expressed in the lemmas, whereas, expression of LtMADS1 was detected in the lemma, stamen and carpel primordia even at anther + stage, the latest stage analysed in this study. Expression of

LtMADS1 and LtMADS2 in lodicule and palea primordia could not be determined at this stage. The expression of LtMADS2 in the lemma and its exclusion from reproductive tissue agrees with the expression of ZAP1 (Mena *et al.*, 1995), the LtMADS2 homologue from maize. The highest level of LtGAMYB expression was within the stamen primordia correlating with the probable high level of GA's in stamen tissue (Pharis and King, 1985). Expression of LtLFY was not detected in organ primordia which initiated from the floret meristem. Expression of CDC2aLt was not analysed in organs derived from the floret meristem.

Expression of LtMADS1, LtMADS2, LtGAMYB, CDC2aLt and LtLFY were all detected in provascular tissue in the central core of shoot apex at the advanced double ridge stage. LtMADS1, LtMADS2, LtGAMYB and CDC2aLt were also expressed in provascular tissue within developing lemma and glume primordia.

Several contrasting expression patterns were observed (see Table 7.1). During vegetative development LtMADS1 was expressed in an epidermal/subepidermal cone at the edge of the shoot apex (apical dome and leaf primordia), whereas expression of LtMADS2 was restricted from the leaf primordia and the expression of LtGAMYB was restricted from the region between the apical dome and the basal leaf primordia. Next, the early up-regulated expression of CDC2aLt, LtMADS1, LtMADS2 and LtGAMYB at ca. LD III contrasts with the late up-regulated expression of LtLFY at ca. LD XII. Finally, LtMADS1 was expressed in the stamen and carpel primordia at all stages analysed in this study, whereas LtMADS2 was eventually restricted from these organs.

Table 7.1 Summary of CDC2aLt, LtMADS1, LtMADS2, LtGAMYB and LtLFY expression patterns during the vegetative to floral transition in *Lolium temulentum* strain Ceres determined by *in situ* hybridisation.

	Transcripts detected in:								
	VM	**SM**	**FM**	**Gl**	**Le**	**Pa**	**Lo**	**St**	**Ca**
CDC2aLt	+	++	+	+	+	?	?	?	?
LtMADS1	++	++	++	+	+	?	?	+	+
LtMADS2	+	++	+	+	+	?	?	+➤-	+➤-
LtGAMYB	+	++	+	+	+	?	?	++	+
LtLFY	-	++	-	+	++➤-	?	?	-	-

+ indicates expression detected; ++ indicates peak level of expression; +➤- indicates expression detected at times proximal to organ initiation with little or no expression detected during organ development; - indicates no expression was detected and ? indicates expression was not assessed.

VM = vegetative meristem; SM = spikelet meristem; FM = floret meristem; Gl = glume; Le = lemma; Pa = palea; Lo = lodicule; St = stamen; Ca = carpel; ? = not determined.

7.2 *Interaction of LtMADS2 with LtLFY*

Lolium temulentum is the first monocot species in which the mRNA expression of both an AP1-like (LtMADS2) transcript and LEAFY (LtLFY) have been characterised by *in situ* analysis. Expression of homologues of this pair of genes have previously been characterised in *Arabidopsis* (AP1 and LEAFY; Mandel *et al.*, 1992; Weigel *et al.*, 1992;), *Antirrhinum* (*SQUA* and *FLO* respectively; Coen *et al.*, 1990; Huijser *et al.*, 1992), and *Brassica oleracea* (*BoFH* and BoAP1; Anthony *et al.*, 1996). Even though significant sequence similarity exists between the *Lolium* LtLFY and LtMADS2 and the dicot homologues, their temporal and spatial patterns differ significantly: (i) temporally, LtMADS2 is expressed at a low level within the vegetative meristem prior to floral induction, as well as in the spikelet (inflorescence) and floret (floral) meristems after induction, whereas in dicots expression of AP1 homologues is restricted to the floral meristem and is not detected in either the vegetative or the inflorescence meristems. (ii) expression of LtLFY is not detectable until glume/lemma stages ca. 9 to 12 d post-induction, whereas, in *Arabidopsis* LEAFY expression increases within 16 h following LD floral induction, which is significantly before AP1 on day 3 after induction (Yanofsky, lecture at 14th International Congress of Sexual Reproduction, Lorne Australia, 1996). In *Sinapis alba*, LEAFY is expressed within the vegetative meristem with SaMADS C (its AP1 homologue; Menzel *et al.*, 1995) not expressed until LD 7 (Melzer *et al.*, 1995; Menzel *et al.*, 1996). Therefore the temporal sequence of LtLFY and LtMADS2 is opposite to that of their homologues in both *Arabidopsis* and *Sinapis*. (iii) spatially, LtLFY is expressed within the spikelet meristem and in the lemma and glume primordia. Although LtLFY is expressed uniformly within the terminal spikelet site, its expression (and that of LtMADS1 and

LtMADS2) is confined to the dorsal side of the lateral spikelet sites. By comparison, in *Antirrhinum*, FLO is expressed uniformly in the floral meristem and bracts, and in *Arabidopsis*, LEAFY expression is uniform within the floral meristem. In *Lolium*, LtLFY and LtMADS2 are coordinately expressed in the spikelet (inflorescence) meristem whereas in *Arabidopsis* LEAFY and AP1, and in *Antirrhinum* FLO and SQUA, coordinately localise within the floral meristem (Coen *et al.*, 1990; Mandel *et al.*, 1992; Huijser *et al.*, 1992; Weigel *et al.*, 1992).

In the *terminal flower 1* mutant (*tfl1-2*) of *Arabidopsis*, and this mutant's counterpart *centroradialis* (*cen*) in *Antirrhinum*, AP1 and LEAFY expression (SQUA expression implied from data available for FLO) invades the inflorescence meristem causing it to become determinate rather than remaining indeterminate (Gustafson-Brown *et al.*, 1994; Bradley *et al.*, 1996). The inflorescence meristem also becomes determinate in *Arabidopsis* AP1 or LEAFY overexpressors with neither causing the vegetative apical meristem to become floral (Mandel and Yanofsky, 1995a; Weigel and Nilsson, 1995). Apparently, unlike AP1 in *Arabidopsis*, expression of LtMADS2 (and LtMADS1) is not sufficient to transform a spikelet meristem into a floret. Even expression of LtMADS1 and LtMADS2 in combination with LtLFY within the spikelet meristem does not immediately cause terminal floret initiation since up to six florets (flowers) are produced from the spikelet (inflorescence) meristem before a terminal floret is formed. Perhaps the function of *LtMADS1* and *LtMADS2* are more similar to that of *AGL8* (another *Arabidopsis* member of the SQUA family) since both *Lolium* genes and *AGL8* are expressed in inflorescence meristems without converting them to flowers (Mandel and Yanofsky, 1995b). The role of *LtLFY* is also unclear since its expression in spikelet sites (and lack thereof in floret sites) argues against a role in floral meristem

identity and floral determination in *Lolium*. Equally possible, if *LtMADS1*, *LtMADS2* and *LtLFY* have a role in floral determination, perhaps additional factors are required or conversely, an inhibitor of floral development may have to disappear. This is suggested by the fact that in both *Arabidopsis* (AP1 overexpressor) and *Lolium*, expression of AP1-like genes within the vegetative meristem is not sufficient to cause floral meristem initiation.

7.3 *Evidence for Regulation of LtLFY by GAMYB*

In LDPs, floral induction as a result of the LD photoperiod is generally believed to involve the gibberellin class of plant hormones since, (i) LD floral induction correlates with an increased level of endogenous GA's and (ii) GA's are generally effective at inducing flowering in LDP's grown under SD conditions. Additionally the *Arabidopsis* mutant *ga1-3*, with very reduced GA status, does not flower under SD's (Wilson *et al.*, 1992), whereas *spindly*, a mutant appearing to have a constitutive GA response, flowers early (Jacobsen and Olszewski, 1993).

In *Lolium*, GA's have been shown to induce flowering under completely non-inductive conditions (Evans *et al.*, 1990). The up-regulated expression of LtGAMYB during the vegetative to floral transition may indicate that this gene mediates GA up-regulated gene expression in florally induced shoot apices similar to its role in GA-mediated signal transduction in cereal aleurone (Gubler *et al.*, 1995). Furthermore, this GA-induced MYB may in turn activate LtLFY expression and thus regulate flowering. A number of observations support this proposal: (i) the up-regulated expression of LtGAMYB during the vegetative to floral transition; (ii) induction of flowering with exogenously applied GA's, which also induce LtGAMYB expression in the endosperm; (iii) the expression of LtGAMYB prior to that of LtLFY, and (iv) the possibility of GAMYB binding to a specific motif in the

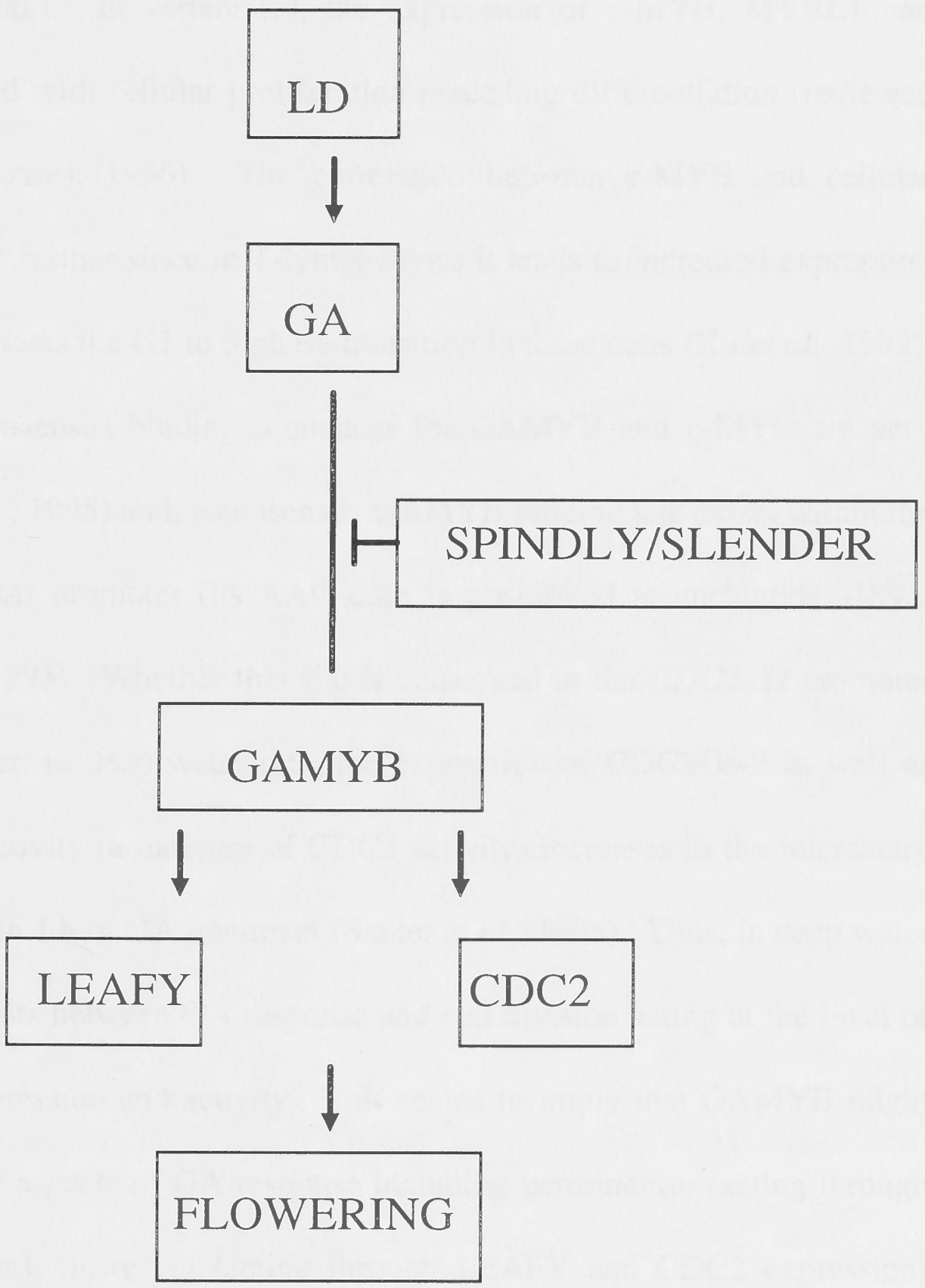

Figure 7.2 Hypothetical role of GAMYB in the activation of LEAFY and CDC2 expression during flowering.

LEAFY promoter.

7.4 *Additional Interactions Between Flowering Genes*

Not only might LtGAMYB transactivate the expression of LtLFY, but perhaps also CDC2aLt. In vertebrates, the expression of c-MYB, MYBL1 and MYBL2 is correlated with cellular proliferation preceding differentiation (reviewed in Martin and Paz-Ares, 1996). The correlation between c-MYB and cellular proliferation extends further since in T-lymphocytes it leads to increased expression of CDC2 which activates the G1 to S phase transition in these cells (Ku *et al.*, 1993). Interestingly, the consensus binding sequences for GAMYB and c-MYB are very similar (Gubler *et al.*, 1995) and, a consensus GAMYB binding site exists within the *Arabidopsis CDC2aAt* promoter (its AAC core is positioned at nucleotide -1895; Chung and Parish, 1995). Whether this site is conserved in the *CDC2aLt* promoter is unknown, however, in deep water rice, the expression of CDC2Os-2 as well as histone H1 kinase activity (a measure of CDC2 activity) increases in the intercalary (leaf) meristem within 1 h of GA treatment (Sauter *et al.*, 1995). Thus, in deep water rice a correlation exists between GA response and cell division acting at the level of increased CDC2 expression and activity. This seems to imply that GAMYB might be involved in many aspects of GA response including germination (acting through α-amylase expression), flowering (acting through LEAFY and CDC2 expression) and elongation growth (acting through CDC2 expression). Besides GAMYB, the expression of other MYB genes in petunia petals is also GA-responsive (Martin and Paz-Ares, 1997).

Other transcriptionally active genes cloned in this study may also interact with each other. This study has shown that transcripts of transcriptional regulators

from three families, LEAFY, MYB and MADS-box increase within the *Lolium* shoot apex during the vegetative to floral transition. Expression of some of these genes display overlapping patterns of temporal and spatial localisation. In *Arabidopsis* and *Antirrhinum*, the ability of plant MADS-box proteins to form homo- and heterodimers has previously been elaborated (Goto and Meyerowitz, 1994; Davies *et al.*, 1996; McGonigle *et al.*, 1996; Riechmann *et al.*, 1996). Given the overlapping expression patterns for LtMADS1 and LtMADS2, their protein products may interact. Perhaps too, the putative truncated protein produced by differential splicing of the LtMADS2 pre-mRNA may provide additional regulation either as a superactive or inactive competitor for dimerisation with full length LtMADS2 or LtMADS1 protein. In addition to LtMADS2, differential processing of CDC2aLt pre-mRNA may also be physiologically important. As an aside, such alternative processing could play an important role during the vegetative to floral transition. In this regard, it is interesting that one of the early flowering mutants, *fca* of *Arabidopsis*, displays sequence similarity to the *SXL* (*SEX LETHAL*) gene from *Drosophila* and this latter gene has been shown to regulate pre-mRNA splicing (Wilson and Dean, 1996).

MADS-box proteins may also form ternary complexes with quite unrelated proteins. In yeast and animal systems, one of the more intimate interactions occurs between the MADS-box and homeobox families of transcription factors (Grueneberg *et al.*, 1992; Olson *et al.*, 1995). To date interactions between members of these families have not been elaborated in a plant system, although two likely candidates might include: (i) KNOTTED-1 (KN-1) or KNOX3 (Smith *et* al., 1992; Jackson *et al.*, 1994) interacting with LtMADS1 and/or LtMADS2 within the vegetative, spikelet or floret meristems in monocots, and (ii) BELL1 might interact

with AGAMOUS or other MADS-box genes expressed during ovule development in *Arabidopsis* (Ray *et al.*, 1994; Reiser *et al.*, 1995). The up-regulated expression of LtMADS1, LtMADS2 and of *Lolium* KN-1-like genes (see Figure 7.3) indicate these genes are coordinately expressed in time. Additionally, a role for KN-1-like genes in flowering has been demonstrated for a mutant of a barley homologue of this gene, *hooded* (*HvKNOX3*), which causes a homeotic transformation within the floret producing an ectopic floret site on the lemma (Müller *et al.*, 1995). An analogous phenotype of floral meristems produced on the surfaces of leaves is caused by overexpression of this gene in tobacco (Müller *et al.*, 1995). This contrasts the ectopic production of vegetative meristems through overexpression of KN-1 also in tobacco (Sinha *et al.*, 1993). If the expression pattern of maize KN-1 (Smith *et al.*, 1992; Jackson *et al.*, 1994) is the same in *Lolium*, then KN-1 protein would co-localise with LtMADS1 and LtMADS2 in the epidermal and subepidermal layers of the shoot apex, spikelet and floret meristems. Perhaps the boundary between KN-1 and, LtMADS1 and LtMADS2 may be functionally important. Possible interactions between meristem identity, organ identity and even flowering time genes remain to be explored.

7.6 *Functional Determination of Genes Isolated in This Study*

This study showed that LtMADS1, LtMADS2, CDC2aLt, LtGAMYB and LtLFY are all up-regulated following LD floral induction within the *Lolium* shoot apex, suggesting each may have a role during the vegetative to floral transition. Mutant analysis and expression of these genes in transgenic plants are two methods whereby insight into their functions may be achieved.

By analysing the expression patterns for LtMADS1, LtMADS2 and LtLFY within mutants of barley, rice or corn exhibiting altered late vegetative or

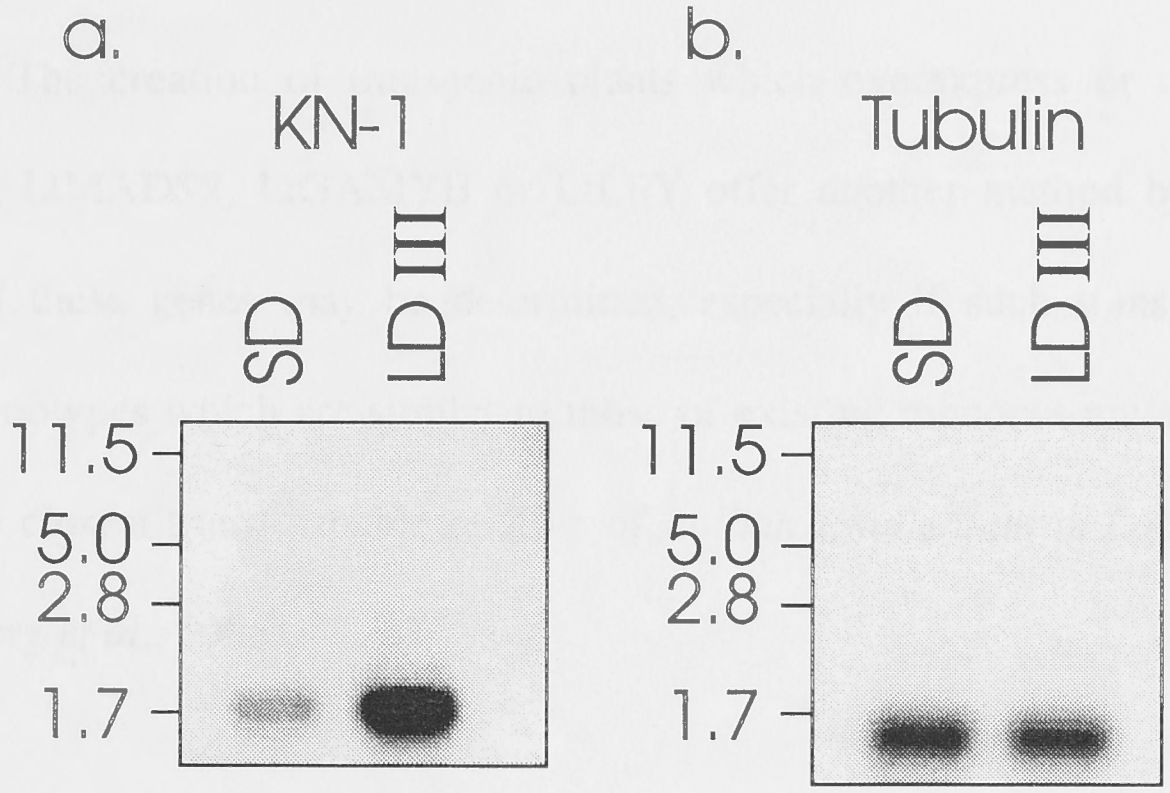

Figure 7.3 Virtual northern blot analysis of vegetative SD and florally induced LD III PCR-based cDNA (24 h after the end of the single LD) from shoot apices hybridised with (a) the maize KNOTTED-1 cDNA (generous gift of Dr S. Hake, USDA-UC Berkeley Plant Gene Expression Center, Albany, CA, USA), or (b) a fragment of a *Lolium* tubulin cDNA (see Table 2.3.4) to compare loading between lanes. Sizes in kbp are indicated to the left.

spikelet morphologies insight into their roles might be obtained. For example, expression of these genes would likely be altered in the barley mutants *branched ear* (*be*) or *branched5* (*brc5*) (Bossinger *et al.*, 1992). These mutants exhibit a similar phenotype to the *Arabidopsis cauliflower / apetala1* double mutant in that each spikelet meristem in these barley mutants is transformed into a new head rather than becoming determinate.

The creation of transgenic plants which overexpress or underexpress LtMADS1, LtMADS2, LtGAMYB or LtLFY offer another method by which the function of these genes may be determined, especially if such transgenic plants display phenotypes which are similar to those of existing monocot mutants.. In this respect, the closest transformable relative of *Lolium temulentum* is *Lolium perenne* (Spangenberg *et al.*, 1995).

Conclusions

Several genes were cloned by homology and found to be up-regulated during and after the vegetative to floral transition within the *Lolium* shoot apex. Three of these genes, *LtMADS1*, *LtMADS2* and *LtLFY*, are homologues of floral meristem identity genes in *Arabidopsis* (*AP1* and *LEAFY*) and *Antirrhinum* (*SQUA* and *FLO*). However, their temporal and spatial patterns of expression contrast significantly with those of their dicot homologues, which may imply that the roles of these genes have also diverged.

Potential for cell division following LD induction was examined by immunolocalisation and *in situ* hybridisation with a CDC2aLt probe. Expression of this gene was increased within 48 h of the LD, corresponding with the increased rate of cell division observed at this time by Ormrod and Bernier (1990). However, it was unable to detect the early transient increase in cell division observed by Jacqmard *et al.* (1993).

Finally, expression of a gene involved in GA signal transduction, *LtGAMYB*, was found to increase in the shoot apex after floral induction. In *Lolium* given that (i) the florally inductive LD enhances LtLFY and LtGAMYB expression, (ii) GA's induce flowering and (iii) LD induces increases in the level of GA's, I propose that LtGAMYB responds to this increased level of GA by activating the expression of LtLFY.

The timeline of events occurring during LD induction of flowering has been extended significantly by this study, however, the functions of *LtMADS1*, *LtMADS2*, *LtLFY* and *LtGAMYB* in *Lolium* remain to be determined. Their functions might be resolved by analysing their expression patterns in existing floral mutants in monocot species, or by antisense and oversense expression of these genes

in transgenic *Lolium* plants. All genes presented in this study were up-regulated suggesting they may be involved in activating the floral program (floral promoters), however, down-regulated genes involved in repression of the vegetative program (floral inhibitors) may also be important during this transition. Fragments of some down-regulated genes have been isolated in a subtractive approach of florally induced against vegetative shoot apices on LD III (not presented). Characterisation and determination of the function of these genes will be the basis of future investigations.

In summary, results of this study (i) show that homologous genes are involved in the vegetative to floral transition in both monocots and dicots, (ii) suggest a mechanism whereby GA may activate LEAFY expression, and (iii) present a framework into which additional genes involved in the vegetative to floral transition in *Lolium* and other monocot species may be placed.

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Appendix I

IN SITU HYBRIDISATION WITH NON-RADIOACTIVE PROBES

All *in situ* hybridisations described in this thesis were performed using this protocol. It has been modified from the protocol of Dr G.N. Drews (unpublished), which was modified from that of Dr D. Oppenheimer. References relevant to this procedure are listed below.

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TABLE OF CONTENTS

I. FIXATION, EMBEDDING AND SECTIONING

A. FAA Fixation:

A variety of fixation procedures have been attempted with the best signals obtained for tissue fixed in FAA. Tissue fixed in paraformaldehyde is almost as good and that fixed in glutaraldehyde gives no signal.

Materials:

Use bulk 95% or 100% ethanol except for the 100% ethanol steps.
For the 100% ethanol steps, use high quality ethanol (e.g., Gold Seal) or 100% bulk ethanol dried over Molecular Sieves (Sigma M-6141).
Glacial acetic acid
37% formaldehyde
small vials: 5 or 20 mL glass scintillation vials (e.g., S/P Baxter #R2550-14)

1. Fixative:

	% in Mixture	Amount
Ethanol	50.0%	50 mL
Acetic Acid	5.0%	5 mL
Formaldehyde	3.7%	10 mL of 37%
Water	41.3%	35 mL
		100 mL

2. Place 10-15 mL of cold fixative into 20 mL scintillation vials and chill on ice. (Labels may be easily written in pencil on white paper, placed in the vials and retained throughout the subsequent dehydration and embedding steps.)

3. With a sharp, cold, razor blade cut (do not squash) the tissue and immerse immediately in cold fixative. The volume of fixative must be large relative to the volume of tissue being fixed.

4. Place tissue/fixative under vacuum for 15 minutes (on ice to prevent flashing of the fixative). Pull the vacuum very slowly. This step removes the air from the tissue replaces it with fixative. After 15 minutes, slowly release the vacuum.

5. Following vacuum infiltration, store the tissue in the fridge until ready to proceed through the dehydration steps (e.g., for time course sampling, strong signal and low background is observed even after 1 month).

B. Dehydration

The tissue must be completely dehydrated before clearing with xylene since water and xylene are immiscible. Tissue is also stained with Eosin Y during these steps to help orient tissue during sectioning.

Materials:

Use bulk 95% or 100% ethanol except for the 100% ethanol steps.
For the 100% ethanol steps, use high quality ethanol (e.g., Gold Seal) or 100% bulk ethanol dried over Molecular Sieves.
Xylene
Eosin Y; Eosin Yellow (Manufacturing Chemists #B286)

1. Remove fixative and begin dehydration steps by half filling the vial with 50% ethanol (in distilled water). Incubate 4 h at room temperature.

2. Every 4 h (approximately, longer incubations (days) cause no harm) proceed through stepwise changes of 60%, 70%, 80%, 90%, 95% and three changes of 100% ethanol. The apices are stained overnight using Eosin Y (0.1%) stain in 95% ethanol. To prevent too much destaining, the 100% ethanol changes are performed during the following day and apices left in 25% Xylene (in ethanol) the following night.

C. Clearing

The tissue must be permeated with xylene since paraffin is immiscible in ethanol. The tissue does not destain (Eosin) in xylene.

Materials:

High quality ethanol.
Xylene

1. Remove the 100% ethanol and replace with 25% xylene: 75% ethanol. Incubate at room temperature 4 h.

2. Repeat for the following xylene:ethanol (v:v) solutions:
50% xylene:50% ethanol
75% xylene:25% ethanol

3. Remove the 75% xylene and replace with 100% xylene. Incubate at room temperature for 4 h.

4. Perform a total of three changes of 100% xylene.

D. Infiltration:

Materials:

Paraplast Plus Tissue Embedding Medium (Oxford Labware)
Molten Paraplast Plus. Place a beaker full of Paraplast chips in an oven at 60°C well in advance; it will take >5 h to melt. Do <u>not</u> exceed 62°C.

1. Add a few chips of Paraplast to each vial. Incubate at room temperature for 4 h.

2. Repeat until xylene solution is saturated with wax at room temperature. Leave O/N.

3. The next morning place vials at 60°C for 4 h.

4. Remove half of the wax/xylene mixture and replace with molten wax. Swirl to mix. Incubate at 60°C for 4 h.

5. Repeat step 4 twice.

6. Remove wax/xylene solution and replace with molten wax. Swirl to mix. Incubate at 60°C for 4 h.

7. Repeat step 6 at least 6 times.

E1. Pouring Boats:

Materials:

Molten Paraplast Plus
Index cards (3 X 5)
Hot plate

1. Fold an index card so that it has raised edges of about 2 cm along its length and about 4 cm along its width. Fold a diagonal line across the rectangle at each corner. To form a box, bring the sides together bending the corners around the width, then overfold the flap at each end of the box.

2. Place the boat on a hotplate set at 70°C.

3. Pour the infiltrated tissue (and label) into the boat.

4. Fill the boat with molten Paraplast Plus.

5. With warm dissecting needles, arrange the tissue into a regular array with at least 2 cm between each tissue piece as well as between the tissue and the wall of the box. Upon sectioning, the tissue face parallel to the bottom of the box will be parallel to the blade edge.

6. Remove the boat from the hot plate and float on a bath of ice water. Once solid completely submerge it and allow the wax to harden completely.

7. Store blocks at room temperature.

or E2. Placing Small Tissue Pieces Into Plastic Molds

Materials:

Molten Paraplast Plus
Peel-a-way disposable plastic tissue embedding molds (Polysciences Inc., #18985)
Hot plate

1. Prewarm the plastic mold on a hot plate set at 70°C, then half fill it with molten wax.

2. Using a Pasteur pipette which has been warmed by passing it through a flame, draw a desired tissue piece into the pipette and expel it into the mold.

3. With warm dissecting needles (18.5 G needles on 1 mL syringes also work well) orient the tissue, carefully remove the mold from the hotplate and allow to cool briefly. It is important that the wax is viscous enough that the tissue does not move when molten wax is added, yet has not solidified so that the additional layer of wax melds with the existing layer.

NB: The tissue is oriented, on a metal block heated to 65°C, visualising the mold under a dissecting microscope. If the wax begins to solidify before the tissue is oriented it is remelted at 57 to 62°C before attempting to rearrange the tissue.

4. Let the wax harden completely. Molds are left O/N at room temperature or are immersed in ice water once the wax has firmed.

F. Coating Microscope Slides With Vectabond Reagent:

Vectabond-coated slides are preferred to other coatings for adhesion of the sections to the slides. Steps 2 through 6 are derived directly from Vectabond data sheet (Vector Laboratories, Burlingame, CA).

Materials:

Microscope slides
Chromerge
Vectabond Reagent (Vector Laboratories, Burlingame, CA)
Staining dishes with slide holders

1. New slides are washed in detergent, rinsed, soaked in Chromerge overnight and rinsed well with running distilled water. Slides can thereafter be dried in a dust free environment, or after passing slides through three changes of acetone, coated immediately with Vectabond Reagent. Any traces of water in the coating solution will cause spots to form on the slides.

2. Clean, dry slides are rinsed for 5 minutes in acetone.

3. The entire contents of one bottle (7 mL) of Vectabond Reagent are added to 350 mL of acetone. This is sufficient to coat 500 slides.

4. The acetone-rinsed slides are placed in Vectabond solution for 5 min.

5. Slides are removed and dipped several times (without creating bubbles) in nanopure water, the slides are tapped to remove adhering water, then air dried thoroughly in a dust-free environment.

6. Coated slides are stored at room temperature until required. The adherence property of the Vectabond-treated slides is stable for at least one year.

G. Sectioning and Adhesion:

Sections can be cut a few days before use or sections affixed to slides can be stored in the fridge under desiccant for periods of up to a few months.

Materials:

Vectabond-coated slides
Flotation Bath heated to 42°C
Slide warmer set at 45-50°C

1. Wax-embedded tissue is mounted onto a microtome block, then cooled in wet ice.

2. Tissue is sectioned at 8 to 10 µm in ribbons.

3. The paraffin ribbon is cut into segments.

4. To relax and reduce compression in the tissue, float the desired sections/ribbons on distilled water prewarmed to 42°C for >1 min. This may be done in a flotation bath or on a layer of water on top of a coated slide, draining the slide once the ribbon has relaxed.

5. Incubate the slides overnight on a slide warmer set to 45°C to "bake" the ribbon piece onto the slide. This must be done immediately after draining excess water from the slide.

II. PROBE SYNTHESIS

A. The Probes:

Generally two probes are synthesised. The first probe hybridises with the RNA of interest, and is generally called antisense, anti-mRNA, or (+) strand probe. A control probe is also synthesised that will not hybridise with the RNA of interest. The most convenient control is to synthesise an RNA probe in the opposite orientation of the anti-RNA probe. This type of probe is termed sense, sense-mRNA, or (-) strand probe.

McFadden (1995) has recently suggested that some of the G/C rich restriction sites (e.g., Apa I, Sma I and Not I) in commonly used vectors such as pBluescript SK and KS as well as pGEM can result in high background through hybridisation with rRNA. Another useful control is therefore a riboprobe consisting of the multiple cloning site 5' to the cDNA insert.

A typical experiment consists of 10 slides; 8 of which are hybridised with the antisense probe and 2 which are hybridised with the sense probe.

B. Solutions:

1. 0.5 M EDTA (RNase Free)

2. 4 M LiCl (RNase Free)

3. 1 M $MgCl_2$ (RNase Free)

4. 70% and 100% Ethanol (RNase Free)

5. Hydrolysis Carbonate Buffer:

Composition:	60 mM Na_2CO_3 40 mM $NaHCO_3$ pH = 10.2
Amount Required:	<1 mL Make up 100 mL for convenience
Formula for 100 mL:	636 mg Na_2CO_3 (MW = 106) 336 mg $NaHCO_3$ (MW = 84) Increase the volume to 100 mL Check that pH is 10.2

6. 10 X Hydrolysis Neutralisation Buffer:

Composition:	2 M NaOAc 10% Glacial Acetic acid pH = 6.0
Amount Required:	<1 mL Prepare 100 mL for convenience
Formula for 100 mL:	16.4 g NaOAc 10 mL Glacial Acetic Acid Increase vol to 100 mL Check that pH is 6.0

C. Materials:

Boehringer Mannheim RNA Labelling Kit (# 1175 025)
20 mg mL^{-1} RNase-free Glycogen (Boehringer Mannheim # 901 393):

D. Template Preparation:

1. Since T7>T3>SP6 in terms of riboprobe yield, I prefer to clone each insert for probe synthesis in both pBluescript SK and pBluescript KS plasmid vectors. The multiple cloning site within these is reversed, thus both sense and antisense probes can be synthesised using T7 RNA polymerase. If enzymes which generate 3' overhangs must be used, end fill with T4 DNA polymerase.

2. Digest with the appropriate restriction enzyme to linearise the template so that a "run-off" transcript can be generated. Avoid the use of enzymes which generate 3' overhangs; they can act as promoters for the polymerases.

3. Phenol/chloroform extract once, and chloroform extract once.

4. Precipitate the DNA, wash well and resuspend in RNase-free water at about 0.2 $\mu g\ \mu L^{-1}$.

5. Determine the exact concentration of linearised DNA by running the linearised plasmid on a gel against a known concentration of vector.

E. Probe Synthesis:

1. Transcription reaction:

The 20 µL reaction described here should yield ca. 10 µg of DIG-labelled RNA. If more DIG-labelled RNA is required, scale up the reaction proportionally.

(a) Reaction Mix:

H_2O	8 µL
10 X Transcription Buffer (vial 8)	2 µL
NTP Labelling Mix (vial 7)	2 µL
Template DNA	5 µL (1 µg)
RNase Inhibitor (vial 10)	1 µL (20 units)

(b) Add 2 µl of RNA polymerase (SP6, T3, or T7)

(c) Incubate at 37°C for ≥ 2 hours

2. DNase treatment:

(a) Add 2 µL of RNase-free DNase (vial 9)

(**b**) Incubate at 37°C for ≥ 15 minutes

(**c**) Add 1 µL of 0.5 M EDTA to stop the reaction

3. Remove a 1 µl aliquot to confirm that the synthesis reaction has worked. Electrophorese the sample on a 1% agarose gel in TBE (non-denaturing) minigel. Include a DNA size marker; do not bother to run a RNA size marker. If the DNase reaction works, the template band will disappear. If the synthesis works well, a thick RNA band of about the correct size will be observed (DNA and RNA do not migrate equivalently). The gel is non-denaturing, so the RNA band may not be tight. For comparison, the template may be run in an adjacent lane.

4. Precipitate by adding 2.5 µL of 4 M LiCl and 75 µL of ethanol. Mix well and incubate at -20°C for ≥ 2 hours (or -70°C for ≥ 30 minutes). Do not add tRNA carrier because its presence will interfere with monitoring the hydrolysis reaction in step F.1.

5. Centrifuge, wash the pellet in cold 70% ethanol and dry it under vacuum. Resuspend the riboprobe in 100 µL of RNase free H_2O.

F. Alkaline Probe Hydrolysis:

The probes are reduced in size to allow better penetration into the tissue. According to Moench *et al.* (1985), probes with a mean length 70 bases generate higher signals than larger probes (150 bases and larger). The probes are degraded to a mean length of ~100 bases chemically, by incubation in alkali at 60°C.

NOTE: It has been suggested by Dr G.N. Drews that DIG-labelled probes are less stable than [35]S probes. Although this is definitely the case with respect to sensitivity to RNase, I do not believe this is the case with respect to base hydrolysis and therefore use the formula in Cox et al. (1984) to determine hydrolysis time.

1. Hydrolysis reaction:

(**a**) The volume of RNA should be 100 µL

(**b**) Remove a 5 µL aliquot as an unhydrolysed control

(**c**) Add 95 µL of carbonate hydrolysis buffer

(**d**) Mix and incubate at 60°C for the time calculated from the following formula:

$$t = (L_o - L_f)/(K)(L_o)(L_f)$$

L_o = starting length (kb)
L_f = final length (kb)
K = 0.11
Calculated time will be in minutes.

Sample calculation, 1 kb transcript to 100 bases mean size:
(1-0.1)/(0.11)(1)(0.1) = 82 minutes

(**e**) Stop the reaction by adding 10 µL of 10 X Hydrolysis Neutralisation Buffer

2. Precipitate the probes by adding the following:

1 µL 20 mg mL^{-1} Glycogen
1 µL 1 M $MgCl_2$
600 µL Ethanol

Incubate at -20°C overnight.

3. Spin down the probe, wash with 70% ethanol, and resuspend in 50 µL of RNase free TE.

4. Remove 2.5 µL for a gel sample. Electrophorese both (pre- and post-hydrolysis) samples on a 1% TBE (non-denaturing) minigel (as in section E.3). Include a DNA size marker on the gel. The hydrolysed sample will be observed as a smear ranging from 50-300 bases.

G. Probe Quantitation and Storage:

In the protocol of Dr G. N. Drew, it is assumed that in the 10 µg synthesis, the loss is about 50%. Thus, he assumes that the probe concentration is about 100 ng μL^{-1}. To determine the best concentration of probe to use, he does a pilot hybridisation experiment using 1 X, 2 X, and 4 X probe. For the 1 X concentration, he uses 200 ng mL^{-1} per kb of probe complexity, or a 1/500 dilution for a 1 kb probe.

I perform a quantitation of the probe against a DIG-labelled RNA control as per (p28-31 Boehringer Mannheim DIG System User's Guide for Filter Hybridisation 1993).

Materials:

Labelled Control RNA (Vial 5 DIG RNA labelling kit)
Anti-digoxigenin-AP, Fab fragments (Vial 3 DIG Nucleic acid detection kit)
NBT solution (Vial 4 DIG Nucleic acid detection kit)
X-phosphate solution (Vial 5 DIG Nucleic acid detection kit)
Blocking Reagent (Vial 6 DIG Nucleic acid detection kit)
Nylon membrane (Boehringer membranes or Hybond-N^+ Amersham)

Solutions:

1. 10 X Blocking Reagent (10 X BR):

See section V.B for composition and formula
<5 mL required per experiment

2. Buffer 1

See section V.B for composition and formula

3. Buffer 2

Blocking reagent stock solution diluted 1:10 in buffer 1 to achieve a final concentration of 1% blocking reagent.

4. TNM-50 Buffer

See section V.B for composition and formula

5. RNA dilution buffer

Mix DEPC-treated H_2O, 20 X SSPE and formaldehyde 5:3:2 (v:v:v).

Procedure:

1. Make 10-fold serial dilutions of the labelling control RNA and labelled antisense and sense hydrolysed probes in RNA dilution buffer. The concentration of the labelled control RNA is 100 µg mL^{-1}. Dilute 1:5 to a concentration of 20 µg mL^{-1}.

Dilution series for RNA labelled reactions:

	Labelled control RNA starting concentration (20 μg mL^{-1})	Stepwise dilution in RNA dilution buffer	Final Concentration	Total dilution
(A)	20 ng μL^{-1}	2 μL/38 μL	1 ng μL^{-1}	1:20
(B)	1 ng μL^{-1}	5 μL/45 μL	100 pg μL^{-1}	1:200
(C)	100 pg μL^{-1}	5 μL/45 μL	10 pg μL^{-1}	1:2000
(D)	10 pg μL^{-1}	5 μL/45 μL	1 pg μL^{-1}	1:20000
(E)	1 pg μL^{-1}	5 μL/45 μL	0.1 pg μL^{-1}	1:200000
(F)	0.1 pg μL^{-1}	5 μL/45 μL	0.01 pg μL^{-1}	1:2000000

NB: In the long term do not reuse these dilutions as they do not remain at their original concentrations.

2. Mark the membrane lightly with a pencil to identify each dilution. Spot 1 μL of the control dilutions onto a positively charged nylon membrane (use dilutions B-F).

3. Spot 1 μL of each of the sense and antisense hydrolysed probe dilutions onto the nylon membrane.

4. Fix RNA to the membrane by cross-linking with UV light or by baking for 30 min at 120^{0}C (Boehringer Mannheim Nylon Membrane).

5. Wash the membrane in buffer 1 (20 mL per 100 cm^2).

6. Block the membrane in buffer 2 for 30 min (20 mL).

7. Dilute anti-DIG alkaline phosphatase 1:5000 in buffer 2 (10 mL).

8. Incubate the membrane in the diluted antibody for 30 min. The diluted antibody solution must cover the entire membrane.

9. Wash the membrane in buffer 1 twice (20 mL each) for 15 min per wash with slow shaking. Change containers between washes.

10. Incubate the membrane in TM50 for 2 min. This buffer "activates" the alkaline phosphatase which is conjugated to the antibody.

11. Mix 45 μL NBT solution and 35 μL X-Phosphate in 10 mL of buffer 3.

12. Decant the buffer and add the colour substrate solution. Allow colour development to proceed in the dark. The colour precipitate starts to form within a few minutes and the reaction is usually complete within 16 h. Do not shake or mix while the colour is developing.

13. When the desired spot intensity has been achieved, stop the reaction by washing the membrane thrice with 50 mL of water.

14. Compare spot intensities of the control and experimental dilutions to estimate the concentration of the sense and antisense probes.

15. For long-term storage (months), it is essential that the probe be diluted 1:10 (v:v) with hybridisation solution. RNase inhibitor (1-2 µL) can also be added to inhibit possible contaminating RNases.

III. HYBRIDISATION

A. Solutions:

1. Set up 10 staining dishes containing the following ethanol solutions: 100%, 100%, 95%, 85%, 70%, 50%, 30%, 15%, H_2O and H_2O. I make these solutions using DEPC-treated water. If hybridising ≤ 10 slides (small staining dishes) 100 mL of each solution will be required, or 300 mL if hybridising 11-20 slides (large 500 mL staining dishes).

2. 20 X SSPE:

Composition:	0.2 M NaH_2PO_4 20 mM EDTA 3.00 M NaCl
Amount Required:	100 mL per experiment
Formula for 1 L:	27.6 g NaH_2PO_4 175.3 g NaCl 7.4 g EDTA Adjust pH to 7.4, increase vol to 1 L DEPC-treat (0.1%) Autoclave

3. 10 X PBS:

Composition:	1.3 M NaCl 30 mM NaH_2PO_4 70 mM Na_2HPO_4
Amount Required:	200 mL
Formula for 1 L:	76.0 g NaCl 3.6 g NaH_2PO_4 9.9 g Na_2HPO_4 DEPC-treat Autoclave

4. Proteinase K Solution:

PBS is used rather than TE since TE is more difficult to render RNase Free. Additionally, EDTA inhibits PK activity since this enzyme requires Ca^{2+}.

Composition:	Dilute 10 X PBS stock in DEPC-water Prewarm to 37°C before adding enzyme.
Amount Required:	100 mL for ≤ 10 slides 250 mL for 11-20 slides
Formula for 20 mg mL^{-1} stock:	Dissolve 20 mg mL^{-1} Proteinase K powder in DEPC-treated water.

5. 50% (w/v) Dextran Sulphate

Prepare 25-50 mL of Dextran (Pharmacia #17-0340-01) in DEPC-treated water and store in the refrigerator

NB: 50% dextran sulfate is difficult to pipette, especially when cold. Warm briefly (~15 seconds) in a microwave.

6. 10 mg mL^{-1} RNase-free yeast tRNA

Dissolve tRNA (BRL #5401) in DEPC-treated water. Store in the freezer at -20°C.

7. 10 X Blocking Reagent (10 X BR):

See section V.B for composition and formula. Each experiment requires <1 mL.

8. Prehybridisation Solution:

Composition:	50% Formamide 2.5 X SSPE 5% Dextran sulfate 1% Blocking reagent 150 µg mL^{-1} tRNA 500 µg mL^{-1} Poly A (only if hybridising with cDNA clone sequences)
Amount Required:	Use ca. 300 µL per slide for the prehybridisation step and the same volume for the hybridisation step for a surface area of 24 X 50 mm. For convenience, make up 10 mL at a time and store frozen.

Formula for 10 mL:

1.60 mL H_2O
5.00 mL Formamide
1.25 mL 20 X SSPE
1.00 mL 50% Dextran sulfate
1.00 mL 10 X Blocking reagent
0.15 mL 10 mg mL^{-1} tRNA (boiled 5 min)

9. Other Reagents/Materials:

Distilled Formamide (BRL #5515)
Plexiglass boxes were designed with two rectangular slide supports on the base and a plastic lid with an absorbent cloth glued to the lid to keep the chamber moist.

B. Set Up For Experiment:

1. Prewarm 1 X PBS solution to 37°C in a staining dish.

2. Set up staining troughs with alcohol solutions for the dehydration step.

3. Remove prehybridisation solution from the freezer and allow to warm to room temperature.

C. Dewaxing:

1. Place slides in a staining dish slide holder and immerse them in a staining dish filled with xylene, with a small stir bar at the bottom.

3. Stir for 10 minutes just fast enough to mix the solution a bit.

4. Repeat with fresh xylene.

N.B. If processing several racks of slides, the same two xylene solutions may be used.

D. Hydration:

1. Remove slides from the xylene and place them into 100% ethanol (95% ethanol will not work). Dip up and down ca. 15 times until the "streaks" go away.

2. Repeat with fresh 100% ethanol.

3. Process the slides through the following ethanol solutions: 95%, 85%, 70%, 50%, 30%, 15%, H_2O and H_2O (all in DEPC-treated water). Once again, dip up and down until the "streaks" go away. Begin with the 95% ethanol and end with the H_2O, in order to hydrate the tissue gradually.

E. Proteinase K Digestion:

Proteinase K is used to partially digest the tissue to allow better probe penetration. This step increases the signal. For *Arabidopsis* floral tissue, the best incubation time is 30-45 minutes, for *Lolium* apices the best incubation time is 30 minutes. Less time gives a weaker signal, and greater time tends to destroy the morphology of the tissue. The timing was calibrated with a proteinase K solution that was prepared just before the incubation.

1. Prewarm the 1 X PBS solution to 37°C in a staining dish.

2. Dilute freshly prepared proteinase K (20 mg mL^{-1}) in prewarmed 1 X PBS solution to a final concentration of 2 µg mL^{-1}.

3. Incubate at 37°C for 30 minutes.

4. After the incubation, remove the slides from the proteinase K solution and place them into a staining dish filled with 1 X PBS. Dip up and down a few times to rinse off the proteinase K.

5. Rinse again in 1 X PBS.

F. Prehybridisation and Hybridisation:

1. Examine the slides and rank them according to quality. Plan to hybridise 8 slides with the anti-mRNA probe and 2 slides with the control probe.

2. Application of prehybridisation solution to the slides:

NB: One hour before use, warm the plexiglass box and moisten the absorbent cloth glued to the lid of the box with 2.5 X SSPE in 50% formamide (i.e., equal osmolarity to the prehybridisation/hybridisation solution). If the box and solution-soaked lid are not warm before use, the prehybridisation solution on the surface of the slides will evaporate.

(a) Pre-warm the prehybridisation solution to 45°C. This makes it easier to spread.

(b) One slide at a time so the tissue is not allowed to dry out, dry the rear surface of each slide with a kimwipe to prevent the prehybridisation solution from being wicked under the slide, then place it in the humid box. Apply the prehybridisation solution in pools across the tissue and spread it to evenly cover each section using a yellow tip. Allow the surface tension of the prehybridisation to draw it between the pools on the slide. Do not touch the sections with the tip.

3. Prehybridise the slides at 42°C for 1-3 h.

4. Boil the required aliquot of probe, chill on ice, spin briefly and add it to the prehybridisation solution. The amount added should be optimised for each probe preparation (see Probe Synthesis section). Mix and let the hybridisation solution sit for a few minutes at 45°C to allow bubbles to rise out of the solution.

5. Remove as much of the prehybridisation solution from the slides as possible by letting it drip off and then wipe the edges and the rear surface back of the slide with a kimwipe. Be careful not to touch the sections. Do not allow the slides to dry out before applying ca. 300 µL of hybridisation solution to the slide. The hybridisation solution should occupy the same area as the prehybridisation solution did thus spreading is not usually necessary.

7. Place the slides back into the humidified box and incubate them overnight at 42°C.

IV. WASH

A. Solutions:

The solutions in these steps as well as those which follow need not be RNase free as duplex RNA is not RNase sensitive.

1. 20 X SSPE (see above)

2. RNase Buffer (optional step):

Composition:	10 mM Tris (7.5) 1 mM EDTA 500 mM NaCl
Amount Required:	100 mL for 6-10 slides 250 mL for 11 to 20 slides
Formula for 500 mL:	5 mL 1 M Tris (7.5) or 0.6 g Tris base 1 mL 0.5 M EDTA or 0.2 g EDTA 14.6 g NaCl Adjust pH to 7.5, increase vol to 500 mL

3. 25 mg mL^{-1} RNase Stock Solution (optional step):

Add RNase Buffer directly to the bottle (Ribonuclease A from Sigma #R-5503) to achieve a concentration of 25 mg mL^{-1}. Store frozen at -20°C. This is a 1000 X stock and will be good for >1 year.

B. Other Materials:

Tupperware boxes for washes
50 or 100 mL staining jars

C. Set Up For Washes:

1. Prewarm 100 mL of the RNase buffer to 37°C (if required).

2. Thaw the RNase stock solution (if required).

3. Prewarm 3 L of 0.1 X SSPE to 55°C (2 L for use before RNase step, 1 L after).

D. Hybridisation Solution Removal:

1. Fill a staining trough with 300 mL and a 2 L Tupperware box with 1 L of 2 X SSPE. Remove as much of the hybridisation solution from the slides as possible by letting it drip off and then wipe around the edges and the rear surface of the slide with a kimwipe. Load slides into slide racks keeping sense and antisense probed-slides separate for this wash. Dip each rack a few times in the staining trough containing 2 X SSPE and transfer the rack to the Tupperware box to wash at room temperature for 15 minutes with gentle agitation.

E. High Stringency Wash:

1. Wash the slides in 1 L of 0.1 X SSPE at 55°C for 40 min with gentle agitation. Repeat this wash for a total of two 40 min washes.

2. Go to the Final Rinse step (**F. 7.**) if not performing the RNase treatment.

F. RNase Treatment (Optional):

Although this step significantly reduces background in a radioactive *in situ* hybridisation experiment, it is uncertain how important this step is for a DIG *in situ*. Apparently DIG-labelled RNA is more sensitive to RNase than ^{35}S-labelled probes. In an experiment using 0, 10 µg mL^{-1}, 50 µg mL^{-1} RNase, it was determined that 0 RNase gave good signal and low background, but that there was very little signal on slides treated with 10 or 50 µg mL^{-1} RNase. Since it does not seem vital, this step has been eliminated.

1. Prewarm 100 mL of the RNase Buffer to 37°C.

2. Thaw the 25 mg mL^{-1} RNase stock, dilute to 2.5 µg mL^{-1} by adding 10 µl to the prewarmed RNase Buffer.

4. Incubate slides in a 37°C hybridisation oven for 30 minutes.

5. After the 30 minute incubation, pour 2.5 µg mL^{-1} RNase directly into sink drain and rinse slides with 0.1 X SSPE (pre-warmed to 37°C).

6. Treat all glassware with DEPC.

7. Place slides into slide rack and wash in 1 L of 0.1 X SSPE at 55°C for 40 min with gentle agitation.

G. Final Rinse:

1. Rinse in 1 X PBS in a staining trough.

2. Proceed immediately to the immunological detection. If the immunological detection is performed the following day, the slides can be stored in 1 X PBS overnight.

V. IMMUNOLOGICAL DETECTION

A. Materials:

Boehringer Mannheim DIG Nucleic Acid Detection Kit (# 1175 041)

B. Buffers:

1. 10 X Buffer 1:

Composition:	1 M Maleic Acid (7.5) 1.5 M NaCl
Amount Required:	50 mL
Formula for 500 mL:	58 g Maleic Acid (MW = 116) 44 g NaCl Adjust pH to 7.5, increase the volume to 500 mL Autoclave

2. 10 X Blocking Reagent (10 X BR):

Composition:	10% Blocking Reagent in Buffer 1
Amount Required:	25 mL
Formula for 100 mL:	10 g of blocking reagent (vial 6) Buffer 1 to a final volume of 100 mL Autoclave and store frozen

3. 1 X Blocking Solution:

Composition:	1% Blocking Reagent in Buffer 1 1% Normal Goat Serum (NGS) (GIBCO BRL #16210-015)

Amount Required: 100 mL for <10 slides
250 mL for 11 to 20 slides

Formula for 250 mL: 25 mL 10 X Blocking Reagent
22.5 mL 10 X Buffer 1
2.5 mL NGS
Adjust to final volume of 250 mL with water

4. 1 M $MgCl_2$

5. BSA Wash solution:

NOTE: The BSA quality is most critical and must be free of endogenous phosphatases (e.g., ELISA quality BSA; Sigma #A-7030).

Composition: 1% BSA
0.3% Triton-X-100
1 X PBS

Amount Required: 800 mL 4 x 200 mL

Formula for 800 mL: 8 g BSA
80 mL 10 X PBS
Adjust the volume to 500 mL and stir for 1 h to dissolve the BSA.
add 2.4 mL Triton X-100
Increase the volume to 800 mL.

6. TNM Buffers

Use TNM-50 to activate the alkaline phosphatase with or without polyvinyl alcohol in the colour reaction. Use TNM-50 as the buffer base if not using polyvinyl alcohol in the colour reaction. Use TNM-5 as the buffer base if using polyvinyl alcohol in the colour reaction.

TNM-5 Buffer:
Composition: 100 mM Tris (9.3)
100 mM NaCl
5 mM $MgCl_2$

Amount Required: ~500 mL

Formula for 500 mL: 50 mL 1 M Tris or 6.1 g Tris base
10 mL 5 M NaCl or 2.9 g NaCl
2.5 mL 1 M $MgCl_2$
Adjust pH to 9.3; increase the volume to 500 mL

TNM-50 Buffer:

Composition:
100 mM Tris (9.3)
100 mM NaCl
50 mM $MgCl_2$

Amount Required:
~500 mL

Formula for 500 mL:
50 mL 1 M Tris or 6.1 g Tris base
10 mL 5 M NaCl or 2.9 g NaCl
2.5 mL 1 M $MgCl_2$
Adjust pH to 9.3; increase the volume to 500 mL

7. TNP Buffer:

This buffer is only necessary if using polyvinyl alcohol in the colour reaction

Composition:
100 mM Tris (9.0)
100 mM NaCl
10% (w/v) Polyvinyl alcohol M_r of 70-100 kDa (Sigma #P-1763)

Amount Required:
~40 mL per set of 10 slides

Formula for 100 mL:
10 mL 1 M Tris
2 mL 5 M NaCl
10 g PVA

Add Tris and NaCl; adjust pH to 9.3 and adjust the volume to 100 mL
Dissolve PVA at 90°C
Cool to room temperature before making PVA Colour-Substrate Solution

8. TE (pH 8.0):

Composition:
10 mM Tris (8.0)
1 mM EDTA

Amount Required:
500 mL

Formula for 500 mL:
5 mL 1 M Tris
1 mL 0.5 M EDTA
Adjust pH to 8.0; increase the volume to 500 mL
Autoclave

9. Staining troughs containing 70%, 100% and 100% ethanol.

C. Blocking:

1. Place slides in staining troughs containing 1 X Buffer 1. Equilibrate 5 minutes. Repeat once.

2. Remove buffer 1 and cover slide rack with 1 X Blocking Reagent. Incubate at room temperature with gentle agitation for 1 hour.

D. Antibody Reaction:

The optimal antibody dilution will probably vary between probes. Try a 1:3000 dilution first. If the signal is too weak, try a lower dilution. If it is very strong, try a higher dilution to save antibody.

1a. Dilute the anti-DIG-AP conjugate (vial 3) in an aliquot of Block Solution (directly from the trough used in the blocking step). If using slide mailers, make up 15 to 16 mL for each container. The antibody reaction can also be performed in a moist chamber (use water to moisten the lid and not 50% formamide) with 500 µL of diluted antibody added to the surface of each slide. Again, dry the rear surface of each slide with a kimwipe and apply diluted antibody immediately since background is increased if slides are allowed to dry. Be certain all tissue is covered with antibody solution. Incubate antibody-soaked slides at room temperature in a moist chamber for 1 to 3 h

or

1b. Place slides in slide mailers and add antibody solution. Incubate at room temperature for 1-3 hours with slow rotation. .

E. Antibody Washes:

1. Remove slides from the antibody solution (or remove the antibody solution from the slides) and transfer them to a staining rack containing either BSA Wash Solution or the original block solution. Work fast so that the antibody does not dry on the slides. Once all slides are in the rack, incubate for 10 min at room temperature with gentle agitation.

2. Transfer the rack to a new staining trough with enough BSA Wash Solution to cover the slides. Troughs must not be reused in subsequent wash steps or a high background will result.

3. Repeat step 2 thrice for a total of 4 washes in BSA Wash Solution.

F.1 Colour Reaction <u>Without</u> Polyvinyl Alcohol:

The colour reaction can be performed in one of two ways, either with or without polyvinyl alcohol.

1. Wash slides in TNM-50 Buffer in a stain trough for 5 minutes at room temperature with gentle agitation.

2. Repeat step 1.

3. Prepare Colour-Substrate Solution just prior to use. Store it in the dark. To perform the colour reaction in slide mailers, 15-16 mL of this solution will be required per container. Ten slides can also be stained (back to back) in a 50 mL staining jar (40 mL of solution). For 40 mL, mix the following:

40 mL TNM-50 Buffer
135 µl X-phosphate-solution (vial 5)
173 µl NBT (vial 4)

4. Place slides in slide mailers (or staining jar) and add the Colour-Substrate Solution. Incubate in the dark in a 30°C oven without shaking.

F.2 Colour Reaction with Polyvinyl Alcohol:

It has been reported that PVA decreases the incubation time and increases the cellular localisation of the colour reaction by slowing the diffusion of reaction intermediates (see De Block and Debrouwer, 1993). In my experience, PVA does not significantly decrease the incubation time. However, I have observed that PVA does improve cellular resolution.

1. Wash slides in TNM-50 Buffer for 5 minutes at room temperature with gentle agitation.

2. Repeat step 1.

3. Prepare the PVA Colour-Substrate Solution just prior to use. Store it in the dark. To perform the colour reaction in slide mailers, 15-16 mL of this solution will be required per container. Ten slides can also be stained (back to back) in a 50 mL staining jar (40 mL of solution). For 40 mL, mix the following:

40 mL TNP Buffer
200 µl 1 M $MgCl_2$
135 µl X-phosphate-solution (vial 5)
173 µl NBT (vial 4)

4. Place slides in slide mailers (or staining jar) and add PVA Colour-Substrate Solution. Incubate in the dark in an oven at 30°C without shaking.

G. Monitoring the Colour Reaction:

1. For a typical probe, monitor the reaction progress beginning at about 6 hours. If the signal is expected to be very strong, begin monitoring at 1 hour.

2. To monitor the reaction progress, choose a test slide, rinse it with TE (pH 8.0), wipe off the back of the slide, and observe it using an old microscope. Never observe unmounted slides using a good microscope!! Put the test slide back into the colour solution if a longer incubation is required.

3. When the colour reaction is complete, wash the slides for 5 minutes with TE (pH 8.0). Repeat thrice. For slides stained in PVA Colour-Substrate Solution, the PVA must be thoroughly washed off.

4. Dehydrate for 15 s in each of 70% ethanol and two changes of 100% ethanol. Allow to air dry overnight in a dust free environment (e.g., covered in foil).

VI. SLIDE MOUNTING

A. Materials/Solutions:

1. Xylene in a coplin jar

2. Coverslips

3. Cytoseal or Permount or DePex mounting medium

B. Procedure:

1. Mount slides in a fume hood. Process 1 slide at a time because the colour fades in xylene.

2. Dip the dry slide in xylene until the streaks go away. Let sit for another 10 seconds.

3. Remove the slide and place it on a clean sheet of aluminium foil. Quickly (before the xylene evaporates) add 1-3 drops of mounting medium to the slide and gently lower a coverslip onto the slide.

4. Squeeze out air bubbles by pressing down gently on the coverslip.

5. If you wish to observe the slides at this point, use the old microscope.

6. Place slides on a slide warmer set at 42°C overnight to harden the mounting medium. Before observing the slides, using a razor blade, scrape off and cut away any excess mounting medium. Never use the good microscope until step 6 has been completed.

Appendix II

IMMUNOLOCALISATION WITH ALKALINE PHOSPHATASE 2^{O} ANTIBODY

TABLE OF CONTENTS

I. FIXATION, EMBEDDING AND SECTIONING

The protocols for fixation, embedding and sectioning were identical to those detailed in Section I of Appendix I.

II. BLOCKING AND INCUBATION WITH PRIMARY ANTIBODY

A. Controls:

The protein of interest was localised with an antibody (monoclonal being better than polyclonal) raised against that protein or an antigenic epitope within the protein. Negative controls included preimmune serum, or immune serum which had been blocked with the competitor peptide to which the antibody was raised.

B. Solutions:

1. Set up 10 staining dishes containing the following ethanol solutions: 100%, 100%, 95%, 85%, 70%, 50%, 30%, 15%, 1 X PBS and 1 X PBS. For ≤ 10 slides (small staining dishes) 100 mL of each solution will be required and 250 mL for 11-20 slides (large 500 mL staining dishes).

2. 10 X PBS:

Composition:	1.3 M NaCl 30 mM NaH_2PO_4 70 mM Na_2HPO_4
Amount Required:	200 mL
Formula for 1 L:	76.0 g NaCl 3.6 g NaH_2PO_4 9.9 g Na_2HPO_4 Autoclave

3. Proteinase K Solution:

PBS was used rather than TE since EDTA inhibits PK activity since this enzyme requires Ca^{+2}.

Composition:	Dilute 10 X PBS stock in distilled water Prewarm to 37°C before adding enzyme.
Amount Required:	100 mL for ≤ 10 slides 250 mL for 11-20 slides
Formula for 20 mg mL^{-1} stock:	Dissolve 20 mg mL^{-1} proteinase K powder in distilled, autoclaved water.

4. PBT

Composition:	0.1% Tween 20 in 1 X PBS
Amount Required:	100 mL for ≤ 10 slides 250 mL for 11-20 slides
Formula for 1 L:	100 mL 10 X PBS stock solution Dilute to 1 L with distilled water Add 1 mL Tween 20

5. BSA Block solution:

NOTE: The BSA used is very critical. If antibody binding is detected with an alkaline phosphatase-conjugated antibody, BSA which is free of endogenous phosphatases (e.g., ELISA quality BSA; Sigma #A-7030) must be used to avoid high background staining.

Composition:	1% BSA 0.3% Triton-X-100 1% Normal Goat Serum (NGS) (GIBCO BRL #16210-015) 1 X PBS
Amount Required:	200 mL
Formula for 800 mL:	8 g BSA 80 mL 10 X PBS Adjust volume to 600 mL and stir for 1 h to dissolve the BSA. Add 2.4 mL of Triton X-100 and dilute to 800 mL. Add 8 mL Normal Goat Serum

6. Other Reagents/Materials:

Plexiglass boxes were designed with two rectangular slide supports on the base and a plastic lid with an absorbent cloth glued to it. The lid was soaked with 1 X PBS to create a moist chamber.
or slide mailers
Glycine
Xylene

C. Set Up for Experiment:

1. Prewarm 1 X PBS solution to 37°C in a staining dish.

2. Set up staining troughs with alcohol solutions for the dehydration step.

D. Dewaxing and Hydration:

The dewaxing and hydration steps were identical to those in sections III C and D of Appendix I, however, the two changes of water at the end of the hydration procedure were substituted for 1 X PBS.

E. Proteinase K Digestion:

Proteinase K is used to partially digest the tissue to allow better antibody penetration. This step increases the signal, however, overdigestion of the sections must be avoided since this treatment will also digest the protein of interest too. For *Arabidopsis* floral tissue and *Lolium* apices, the best incubation time is 10 minutes. Weaker signal is observed with under- or over-digestion. The timing was calibrated with a proteinase K solution that was prepared just before the incubation.

1. To the prewarmed 1 X PBS solution, add proteinase K to a final concentration of 20 μg mL^{-1}. A stock solution of proteinase K (20 mg mL^{-1}) is prepared fresh in each experiment.

3. The digestion is allowed to proceed at 37°C for 10 minutes.

4. Thereafter, the slides are removed from the proteinase K solution and protease activity blocked with glycine (2 mg mL^{-1}) in 1 X PBS. The blocking step is repeated again.

F. Blocking:

High concentrations of BSA as well as goat serum are used to inhibit non-specific binding of the antibody within the tissue of interest.

1. Examine the slides and rank them according to quality. Plan to immunolocalise the protein of interest on 8 slides and use 2 slides for negative controls.

2. Arrange slides in a staining rack. Wash them for 5 min in PBT.

3. Place the rack in a staining trough containing BSA Block Solution.

4. Incubate at room temperature with gentle agitation for 1 hour.

G. Primary Antibody Reaction:

The optimal antibody dilution will vary between primary antibodies. If an Fab fragment which recognises the protein of interest is available, it will achieve better tissue penetration due to its reduced size. To determine the optimal dilution for an antibody, try a series of dilutions from 1:500 through 1:3000. If the staining is still too intense with the 1:3000 dilution, or too weak with the 1:500 dilution, then raise or lower the dilution accordingly. A slightly lower dilution is usually required for immunolocalisation than for western blotting.

NB: Never freeze/thaw an antibody repeatedly. This drastically reduces its activity. Thawed sera preserved with 0.1% sodium azide can be stored at 4°C for several years. Store thawed commercial antibodies as recommended by the manufacturer.

1. Dilute the primary antibody and preimmune serum to the desired dilution in BSA Block Solution (directly from the trough in which the blocking step was performed). If performing the antibody reaction in a moist chamber, 500 µL per slide will be sufficient. For slide mailers, 15-16 mL per mailer is needed.

NB: Save enough (i.e., 500 µL per slide if using a moist chamber or 15-16 mL per slide mailer) BSA Block Solution, stored frozen, to enable dilution of the secondary antibody in step IV-B.1.

2a. In a moist chamber, add 500 µL of the diluted antibody to the surface of each slide. This method requires that the rear surface of each slide be dried with a kimwipe (this retains the antibody solution on the upper surface of the slide) before antibody solution is added. Additionally, the slides must not be allowed to dry significantly since this will result in a high background. Incubate the slides at 4°C overnight in the moist chamber.

2b. Place slides in slide mailer and add the appropriately diluted antibody solution. Incubate at 4°C overnight with slow rotation.

III. WASHES

A. Solution:

1. PBT

Formula is provided in section II B.5.

B. Antibody Washes:

1. Transfer the antibody-treated slides to staining trough containing PBT. Work fast so that the antibody does not dry up on the slides. Once all slides are in the rack, incubate for 5 min at room temperature with gentle agitation.

2. Transfer the rack to a new staining trough with enough PBT to cover the slides. Incubate for 5 min at room temperature with gentle agitation. Troughs must not be reused in the wash steps or a high background will result.

3. Repeat step 2 thrice for a total of 4 washes.

IV. INCUBATION WITH SECONDARY ANTIBODY

A. Solutions:

1. BSA Block solution:

See section II B for the formula.

6. Other Reagents/Materials:

Affinity purified alkaline phosphatase-conjugated goat anti-rabbit IgG (Promega, #S3731)

B. Secondary Antibody Reaction:

A secondary antibody, raised against IgG from the species in which the primary antibody was raised, conjugated to alkaline phosphatase is recommended in plant tissue. High background resulting from tissue autofluorecence, or endogenous biotin or peroxidases, may prevent detection of a successful primary antibody/antigen complexes via fluorescein-, streptavidin- or horseradish peroxidase-conjugated secondary antibodies respectively.

The optimal antibody dilution will differ between secondary antibodies. Again, the optimum dilution must be determined. Try a series of dilutions from 1:100 through 1:500.

1. Prepare an appropriate dilution of the secondary antibody in an aliquot of BSA Block Solution. If performing the antibody reaction in a moist chamber, 500 µL per slide will be sufficient, whereas for slide mailers, 15-16 mL per mailer is required.

2a. If performing the secondary antibody reaction in a moist chamber, dry and apply the desired antibody dilution as detailed in section II G.2a. Incubate at room temperature for 1 h in the chamber.

2b. Place slides in slide mailer and add secondary antibody solution. Incubate at room temperature for 1 h with slow rotation.

3. Perform wash steps exactly as detailed in section III.

V. COLOUR REACTION AND SLIDE MOUNTING

Successful binding of the alkaline phosphatase-conjugated secondary antibody to primary antibody molecules was detected and slides dehydrated exactly as in section V F and G of Appendix I. Thereafter, slides were mounted as in section VI of Appendix I.